The Groningen LCPUFA study:
associations between neonatal and early postnatal fatty acid status and developmental outcome at 9 years

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Chapter 1

General Introduction

The essentiality of fat was first discovered by Burr (1). The rats in the study were fed different diets, one of which being without any fat. The animals developed symptoms of deficiency such as dermatitis, kidney problems and growth retardation leading to a premature death. The issue of fat essentiality was further studied in humans by, among others, Hansen and Wiese in the 1950ties (2). Specifically the importance of omega-3 fatty acids wasn't recognized until the 1980ties, when Holman (3) detected a deficiency syndrome in a girl maintained on total parenteral nutrition rich in omega-6 fatty acids but low in omega-3 fatty acids. The deficiency symptoms comprised of, among others, periods of numbness, paresthesia, weakness, inability to walk and impaired vision. Since the eighties considerable progress has been made in the understanding of the physiological functions of essential fatty acids (EFA) in animals and humans and their role in chronic diseases. Two of the most important fatty acids for the nervous system are docosahexaenoic acid (DHA) from the omega-3 group and arachidonic acid (AA) from the omega-6 group. Supplementing formula with these LCPUFA has been found to be beneficial in early infancy (especially DHA supplementation), however outcome assessed between the ages of 6 and 24 months usually did not demonstrate differences between supplemented and non-supplemented groups (4). This age period however is known for its insensitivity to reveal effects of exposures during early ontogeny (5), implying that absence of an effect during this period does not preclude a possible effect a later age. Currently no data exist on the effect of LCPUFA supplementation of formula on developmental outcome at school age. Advantages of another form of early nutrition, breastfeeding, at school age have been found consistently, even though recent studies have shown that associated factors, such as maternal IQ and SES may play a role (6-8). This indicates that LCPUFA supplementation of formula may affect developmental outcome at school age.

The effect of DHA and AA supplementation in infants early postnatally on development at age 9 is the subject of the first part of this thesis.
The relation between fatty acid status at birth and development at age 9 is the subject of the second part of this thesis.

This chapter gives a short overview of the role of fatty acids in the human body. The focus lies on fatty acid metabolism, fatty acids in the cardiovascular system and in growth and the role of fatty acids in brain development. The chapter also includes a summary of human brain development and features an overview of the test battery used in the study. It finishes with the questions addressed in the thesis.

1.1 Fatty acid metabolism

Fatty acids consist of a carbon chain skeleton with a carboxyl group at one end. Fatty acids exist in saturated and unsaturated forms, with trans-fatty acids as a special form of industrially saturated fatty acid. The difference between saturated and unsaturated fatty acids lies

in the presence of double bonds in their skeleton. The unsaturated fatty acids have at least one double bond were saturated fatty acids have none. Polyunsaturated fatty acids (PUFA) have a minimum of two double bonds. Different PUFA groups are distinguished, named after the number of the carbon bond at which the first double bond is located counting from the carboxyl end of the molecule. For example: the fatty acids with the first bond at the 3rd carbon atom are known as the ω-3 or the n-3 fatty acids, and the fatty acids with the first bond at the 6rd carbon atom are known as the ω-6 or the n-6 fatty acids. For this thesis the n- notation will be used. PUFA with 20 or more carbon bonds are known as long-chain PUFA (LCPUFA). Each fatty acid has, aside from the systematic and common name, a shorthand notation. The first number is the number of carbon atoms in the molecule, the second number, right after the colon, is the amount of double bonds and the last number is the location of the first double bond counting from the carboxyl group. See Table 1 for a list of the most relevant fatty acids in this thesis.

Table 1.

Common name	Shorthand notation
Linoleic acid	18:2 n-6
α-Linolenic acid	18:3 n-3
Arachidonic acid (AA)	20:4 n-6
Docohexaenoic acid (DHA)	22:6 n-3

Linoleic acid (LA) is the parent fatty acid for the n-6 fatty acids group to which arachidonic acid (AA) belongs and α-Linolenic acid (ALA) is the parent fatty acid for the n-3 fatty acid group to which docosahexaenoic acid (DHA) belongs (9). The parent EFA can be converted into LCPUFA, by alternating desaturation (delta-5 and delta-6 desaturases) and chain elongation, see figure 1 (10). These enzymes are limited and similar for both the n-3 pathway as for the n-6 pathway resulting in a process of competition in which high intake of DHA results in a decrease of AA levels (9).

Omega-3 and omega-6 long-chain fatty acids are conditionally essential, meaning that humans can synthesize these fatty acids de novo from their respective precursor, however due to the limited enzyme availability not in sufficient amounts. This is especially true for DHA. This may be concluded from the dependence of DHA levels in the blood on variations in the diet (11–13). AA seems much more robust to dietary limitations (11,14–16). Sources of AA are meat, eggs and poultry. DHA is mainly consumed via fatty fish.

Trans-fatty acids can be found in baked foods and fast foods and also in lower concentrations in dairy products. They are incorporated in the body tissues and inversely related to LCPUFA status (17,18).

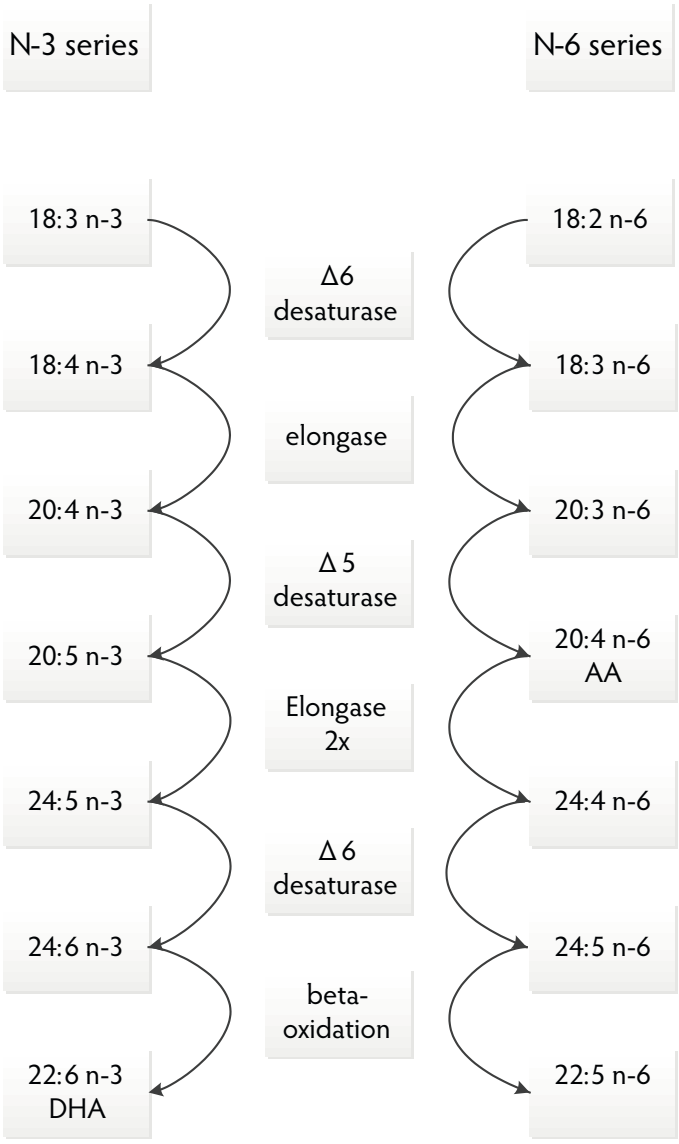

Figure 1. Metabolism and nomenclature of the main LCPUFA's of the n-6 and n-3 series. Both pathways use the same enzymes for desaturation and elongation.

1.2 Fatty acids and the cardiovascular system and growth

There have been numerous studies on the association between omega 3 fatty acids and cardiovascular disease at adult age (19–24). In general these studies suggest cardio protective effects of omega 3 fatty acids, however definitive conclusions cannot be drawn due to suboptimal research designs and the exclusive use of high risk populations. Some of the problems encountered in these large studies were a high level of drop-out, open-label studies, lack of power, lack of control group and problems in checking long-term adherence (25).

Studies on the association between LCPUFA supplementation at early age and cardiovascular indices, such as blood pressure and heart rate, in humans are limited in number and vary in quality. The studies differ in duration of supplementation, fatty acid dosage, and lipid composition. Inconsistent results are reported. Beneficial effects of LCPUFAs on blood pressure and heart rate have been reported by Forsyth et al (26) and Pivik et al (27), but no effects of LCPUFA were found in other studies (28). Asserhoj et al (29) even reported a negative effect.

A limited number of studies has been carried out on the effect of early postnatal LCPUFA supplementation on growth measures and follow-up has not surpassed the age of 3 years. Until that age range no effect was found on head circumference, weight, length and resulting body mass index (BMI) (4). This also implies no negative effects of LCPUFA supplementation on growth in the first three years of life. Breastfeeding on the other hand is related to growth. Having been breastfed is associated with a small but consistent reduction in obesity risk in later childhood (30).

1.3 Other roles of fatty acids in the body

The parent EFA and LCPUFA are important components of cell membranes all through the body. LA has an important role in the skin where it contributes to the skin's barrier function and thereby limits transepidermal water loss. In contrast to LA, only a limited amount of ALA gets stored in the body (31).

EFA make up 20% of human adult dry brain weight, including about 6% for AA and 8% for DHA. AA is especially important as a second messenger in synaptic signal transduction. DHA is a major structural lipid of the retinal photoreceptor outer segment membrane. In the brain it also plays a role in gene expression, apoptosis (programmed cell death) and neuronal development (9,32–35).

LCPUFA can be converted into other useful substances such as eicosanoids (prostaglandins, thromboxanes and leukotriens) that play a role in many bodily systems, primarily in inflammation, immunity and as messengers in the central nervous system. Eicosanoids originating from n-3 LCPUFA differ from eicosanoids originating from n-6 LCPUFA. N-6 eicosanoids are in general pro-inflammatory and n-3 eicosanoids much less so. The bal-

ance between these two groups of eicosanoids has implications for many bodily functions. A detailed overview regarding these implications is beyond the scope of this introduction, however the interested reader is referred to De Caterina and Basta (10) for an overview.

A third important function is the role of LCPUFA in gene expression, LCPUFA induce changes in gene expression playing a role in various processes such as lipid metabolism and transport as well as development of the nervous system (36).

1.4 Brain development

The development of the brain starts shortly after conception with the formation of the neural tube. The first half of gestation is a period characterized by neural proliferation and programmed cell death, once neurons have been formed they migrate to their final destination (37). A large part of the cortical neurons migrate along specialized radial glial fibers that span the entire thickness of the hemisphere (38). Migration peaks between months three and five of gestation (39) and is suggested to be finished around week 30 post menstrual age (PMA).

Migration is also the period during which a large part of the neuronal differentiation takes place, encompassing processes like dendrite and axon formation and the production of neurotransmitters and synapses. A process that continues when the final destination of the neuron has been reached (38). An important role in the process of neuronal migration to the cortex is played by the subplate, a transient structure that develops between the periventricular white matter and the developing cortical plate (40). This structure is said to function alike a 'waiting room', forming a temporary goal of afferent fibers heading for their cortical destination. While present in the subplate the temporary connections already form functionally active circuitries, these most likely play a role in the generation of fetal and neonatal behavior (41,42). The subplate is an important structure between the weeks 24 and 36 PMA after which it begins to decline. Around the age of 6 months postnatal age the subplate will have disappeared (40).

Around week 32 PMA age specific characteristics of the future adult brain can be recognized. Distinct vertical layers (43) are found as well as the major neurotransmitter systems, the most common glia and neuron types (44,45). This is also the period in which the cortical gyri and sulci start to develop (46). From this period until the age of 2 years the brain undergoes a large growth spurt, at the end of which the brain weighs 80% of the adult weight. Important processes during this growth spurt are axonal and dendritic differentiation, axon elimination, synapse formation, myelination and glial cell proliferation (37).

Between the ages of 2 and 5 years the brain grows relatively little, from 80% to 90% of its adult weight (47), this phase is therefore sometimes called the 'plateau phase'. During this phase myelination and synaptic remodeling are particularly active.

Neural connectivity is important for smooth communication between brain regions. Smooth communication depends for a large extent on the integrity and maturity of the brain

white matter tracts. Several studies have evaluated the development of white matter and found a steady increase in the overall white matter volume from childhood to adulthood (48,49). Large regional differences are found. For example in the regional variation in the growth of the corpus callosum, where analyses showed continuous age-related changes in the posterior sections however not the anterior sections (48,50). Differences are also found between the genders, for example in the development of the left inferior frontal gyrus (comprising the pars opercularis, triangularis and orbitalis). Here boys showed a linear age-related increase in the WM volume where girls did not, in the ages between 6 to 17 years (51).

Volumes of gray matter begin to decline in late childhood or adolescence (48,49,52–56). This process occurs first in the sensorimotor areas, followed by association areas and finally occurs in the integration areas such as the superior prefrontal cortex and the posterior parietal cortex. The cerebellum shows a more protracted course, decline beginning around the age of 16 for boys and slightly earlier for girls (57).

This so called 'cortical thinning' is considered to represent two concurrent processes: pruning and myelination. The result of these processes is believed to refine connectivity and enhance the efficiency and fidelity of signal transmission. It is therefore regarded as a marker for maturation (58).

1.5 Fatty acids in brain development

1.5.1 The role of fatty acid status during pregnancy

Fatty acid status of the fetus depends on maternal dietary intake, both during pregnancy and prior pregnancy via maternal fatty acid stores using trans placental transport, and maternal and fetal LCPUFA syntheses (59–61). Trans placental transport is selective for LCPUFA meaning the concentration LCPUFA in the fetal circulation is higher than the concentration in the maternal circulation, a process called biomagnification (62). This process causes the fetal LCPUFA levels to be up to 400% of the maternal LCPUFA levels (63,64). Next to biomagnification the fetus can create its own AA and DHA via synthesization from the respective precursors LA and ALA. Fetal and newborn LCPUFA synthesis is significantly better for AA than for DHA (63,65). For the latter the high requirements appear not to be met until 16 weeks after term (33,66,67). A deleterious effect on fatty acid synthesization is caused by smoking, a study by Agostoni reported a diminished maternal capacity to synthesize AA and DHA in relation to maternal smoking during pregnancy (68).

The fetus requires a high amount of LCPUFA especially during the last trimester of gestation when about 90% of fetal fat deposition takes place (63). To provide the required LCPUFA, maternal fatty acids in plasma rise by 50% during pregnancy, which is brought about by accelerated break down of maternal fat stores (63,69). In general in the western

countries DHA intake is lower than the recommended 450 mg LCPUFA-n3 per day, this in contrary to AA intake which appears to be sufficient. This might indicate that both current nutritional intake as well as established maternal fatty acid stores are not optimally equipped to provide for fetal needs. Evidence suggesting this is found in the slow recovery of maternal LCPUFA status after delivery, which is further retarded if the infant is breastfed, as well as in the lower LCPUFA status of multigravidea in comparison to primigravidea (70). A suboptimal LCPUFA provision during pregnancy could lead to a (relative) LCPUFA deficiency in the fetus at birth.

Studies on the relation between prenatal LCPUFA status and neurodevelopment present mixed evidence. Generally, a positive association is found between prenatal AA and DHA status and neurodevelopmental outcome during early infancy (71–74). After early infancy a subtle yet positive relation is only found between the consumption of fish or DHA and not AA with neurodevelopment in some studies (74–85) while no effects were found in other studies (86–88).

Studies on the effect of neonatal trans fatty acids on neurodevelopment are scarce. Yet the limited evidence points towards a negative association, both at early postnatal age and at 18 months (71,75). The precise mechanisms are as yet unknown however the negative association between trans fatty acid status and LCPUFA status may be partly responsible.

1.5.2 The role of fatty acids in the postnatal period

Breastfeeding is generally accepted as the preferred nutrition for infants. The positive effect of breastfeeding is usually attributed to the nutritional value, however recent studies have shown that associated factors such higher maternal IQ play a large role (6).

Despite the ability of infants to synthesize LCPUFA from their precursors this ability does not suffice to fulfill their needs until 16 weeks after term (33,66,67). This may explain why in the absence of a dietary source of DHA, as was usual in infant formula a decade ago, the erythrocyte and plasma DHA concentrations of infants decrease heavily in the first 4 months after birth (89). Autopsy studies in infants, having received formula's without DHA and AA, have also shown this drop in LCPUFA on a brain level. This difference is more pronounced for DHA levels than for AA levels (90-94)

Animal studies have shown that DHA deficiency during development is associated with decreased cognitive and behavioural performance (95) and that dietary supplementation of either DHA or fish oil in the prenatal and early postnatal phase results in an increase of DHA in brain tissue which seems to be accompanied by a decrease of AA levels (96-100). The functional effects of early DHA and fish oil supplementation in animals is unclear. Some studies report acceleration of visual development (101–103) while others report poorer auditory and motor development (96,98–100,104,105). Interestingly, for learning capacity no differences are reported (97,100).

Several human supplementation studies have been performed, the recent Cochrane review by Simmer (4) reported on 18889 term infants from 15 randomized controlled trials. Despite a large variation regarding type of supplementation, concentration and duration of supplementation no clear or consistent benefit is found regarding formula supplementation with LCPUFA on visual acuity and neurodevelopmental outcome.

1.5.3 The role of fatty acids in the brain

The current knowledge on the role of LCPUFA in the developing brain is still limited. Literature shows that LCPUFA have a general influence on neuronal membranes and on neuronal gene transcription (106,107). In the developing brain LCPUFA accretion starts slowly during early fetal development, were it plays a role in the promotion of proliferation and differentiation of cortical neurons (108,109). During the first two trimesters AA accretion is larger than that of DHA (110,111), during the brain growth spurt in the third trimester, a period characterized by a high LCPUFA requirement, DHA accretion becomes larger than that of AA (33,91,110). At term the brain still contains relatively more AA than DHA(110,111). The continuing process of higher DHA accretion after term results in the adult brain featuring DHA as the main LCPUFA (91,93,110–112).

An animal study by Diau (113) demonstrated that LCPUFA accumulate mainly in the cortical grey matter, predominantly in the synaptic membranes, and to a lesser extent in the white matter, this replicates earlier studies (9,33). The study by Diau also demonstrated that the highest concentrations of LCPUFA are found in the basal ganglia, pre- and postcentral cortices, hippocampus and thalamus, indicating that LCPUFA intake might affect in particular, circuitries involved in sensorimotor integration and memory.

1.6 Developmental tests used in this thesis

1.6.1 Neurological examination with special attention to minor neurological dysfunction

The examination of Minor Neurological Dysfunction (MND) according to Touwen (114) pays special attention to the presence of MND. Essential in the diagnostics of MND is the presence of coherent clusters of signs. Single signs do not have clinical significance, however when they co-occur with other signs within a functional domain they do. The examination is organized into eight functional domains: posture and muscle tone, reflexes, dyskinesia, coordination, fine manipulative ability, associated movements, sensory deficits and cranial nerve functioning. The primary outcome of the neurological examination is

the clinical neurological condition: neurologically normal, simple MND, complex MND or neurologically abnormal. A child is considered neurologically abnormal in the presence of a clear neurological disorder such as cerebral palsy. Complex MND denotes at school age the presence of more than two domains of dysfunction and is the clinically relevant form of MND. In an etiological sense it can be considered a borderline form of cerebral palsy as it is linked to a chain of pre- and perinatal adversities (5,115). Simple MND denotes at school age the presence of one or two domains of dysfunction and is present in about 15-20% of children. It has little clinical relevance and can be regarded as typical but non-optimal brain functioning, in other words as a minor neurological difference and not so much a dysfunction. A child is classified as neurologically normal when no domains are scored as deviant or in case of the isolated presence of a mild dysfunction in reflex activity.

In addition to the classification into distinct categories, we used the optimality concept to summarize neurological condition. For each of the 64 items in the examination an optimal range was identified per age-group (114). The total number of items on which the child scored a value within this optimal range formed the neurological optimality score (NOS) of this child. It should be realized that there is a conceptual difference between normality and optimality, as the range for optimal behavior is narrower than that of normal behavior (116). The NOS may be regarded as a quantitative and more subtle expression of the clinical neurological condition. This also means that the two measures are highly correlated (current thesis rho = -0.691, $p < 0.0001$). The quantitative and precise nature of the NOS makes it an excellent instrument to evaluate subtle deviations in neurodevelopmental outcome. The NOS has previously been used in infancy and pre-school age (117); in the current thesis the NOS principles are applied at the age of 9 years for the first time.

The Touwen assessment has a good intra-rater, inter-rater and test-retest reliability (115). Its construct validity is reflected by the differential relationship for simple and complex MND with prenatal and perinatal adversities: adverse conditions during early life have a weak to moderate relationship with simple MND and a strong correlation with complex MND (5,118). Predictive validity is good, i.e. the severity of MND at 9 years is related to the risk of MND at 12 and 14 years and with learning and behavioral problems at 9 and 14 years (5,119,120).

1.6.2 Examination of cognitive development

The cognitive test battery was designed to give a broad overview of cognitive functioning, focusing on those functions most likely to reflect an effect of LCPUFA supplementation, whilst keeping to time restraints. The earlier mentioned study by Diau (113) showed that LCPUFA was especially present in the basal ganglia, hippocampus, thalamus, cerebellum, precentral, postcentral, prefrontal and occipital cortices, areas that are of importance for intelligence, executive function, attention, learning and memory.

The cognitive test battery consisted of the Wechsler Abbreviated Scale of Intelligence (WASI, (121)), subtests from the Nepsy – A developmental neuropsychological assessment (Nepsy, (122)), subtests from the Test of Everyday Attention-Children's version (TEA-Ch, (123)) and subtests from the Children's memory Scale (CMS, (124)).

The WASI is an intelligence test especially designed for a brief and reliable assessment of full, performance and verbal IQ and is considered a valid screening instrument (125). Because of its brevity it is not recommended to be used as a full individual IQ screening instrument, however it is very useful for scientific purposes focusing on comparisons on group level (126).

The Nepsy (122) is designed to reliably assess neuropsychological functioning of children in the ages between 4 and 12 years old and focuses on five domains: attention-executive function, language, sensorimotor functions, visuospatial functions and learning and memory. Of these scales attention-executive function, language and learning and memory were used in the current thesis.

Attention was assessed using the Tea-Ch (123), a standardized and valid test designed to assess selective attention, sustained attention and switch attention in children aged 6 to 16 years old. Selective attention is considered to be 'a capacity to enhance the processing of particular target characteristics regardless of spatial location', sustained attention is considered to be 'the capacity to maintain a particular processing set over time' and attentional control/switch attention (also called spatial attention): 'the capacity to move attention within space'(127)

To provide additional information on verbal memory the Word Pairs subtest of the Children's Memory Scale was used (124).

At the time of testing no Dutch versions nor Dutch norms were available for the WASI, NEPSY, Tea-Ch and the CMS. These tests were therefore translated to Dutch by the researchers and the original norms were used. This is usually not advised however since the goal of the current thesis does not lie in creating individual profiles of neuropsychological functioning but in comparing groups of individuals having received identical testing and scoring this is considered to be acceptable.

1.6.3 Examination of behavioural development

In order to examine behavioural development three questionnaires were used. Firstly the Child Behaviour Check List (CBCL), secondly the Teacher Report Form (TRF) and lastly a Questionnaire on ADHD. The CBCL and TRF are widely used instruments that assess a broad range of competencies and problems. Reliability and validity are well established (128). The difference between the questionnaires lies in the respondent. For the CBCL this is de parent and for the TRF the teacher. The scales include 120 specific problem items and 20 competence items. The respondent rates the problem items over the past 6 months as 0 = not true, 1 = somewhat

true and 2 very true. These problem items are consecutively scored on statistically derived syndrome scales and on DSM oriented scales. For the current thesis, the syndrome scales were used: aggressive behaviour, anxious/depressed, attention problems, delinquent behaviour, social problems, somatic complaints, thought problems and withdrawn/depressed and additionally on the composite scales: internalizing, externalizing and total problems.

1.6.4 Examination of anthropometry and blood pressure

As part of the anthropometric evaluation weight, length and head circumference were measured. During assessments situated at the hospital, weight was measured using a calibrated radwag scale, body length using a standard stadiometer (seca) and head circumference using a tape measure. During assessments situated at the participants home, weight was measured using an available measuring scale and body length and head circumference using a tape measure. Statistical tests confirmed the lack of differences between the two locations. Consecutively, Body Mass Index (BMI) was determined using international standards taking gender and age into account (129). Blood pressure as well as heart rate was measured immediately following a 15 minute rest period, measurement took place on the left arm with the child sitting on a chair and the arm resting on a table. An automated blood pressure monitor was used (Datascope Accutorr plus) with a small adult cuff (10.6 – 23.9 cm size bladder). This procedure was performed twice during the assessment with an hour in between. Heart rate, systolic and diastolic blood pressure were recorded as the mean of the two readings.

1.7 Specific questions addressed in this thesis

This thesis consists of two main parts. The first part concerns the results of the double blind randomized trial focused on the effect of 2 months of postnatal LCPUFA formula supplementation on neurodevelopment at age 9. For a flowchart of the Groningen LCPUFA project see fig 1 chapter 2. The following research topics will be discussed:

— Is 2 months LCPUFA supplementation beneficial for neuro-motor development, at the age of 9 years.
— Is 2 months LCPUFA supplementation beneficial for cognitive and behavioural development at the age of 9 years
— Is 2 months LCPUFA supplementation beneficial for cardiovascular and anthropometric development, expressed in blood pressure heart rate weight height BMI and head circumference, at the age of 9 years

The second part of this thesis concerns the relation between neonatal fatty acid status and neurodevelopmental outcome at age 9. The following research questions will be evaluated:

— Is there an association between DHA AA and transfatty acid status at birth and neuro-development at age 9. The latter is expressed in neuro-motor, cognitive and behavioral development.

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Chapter 2

The Groningen LCPUFA study: no effect of postnatal long-chain polyunsaturated fatty acids in healthy term infants on neurological condition at 9 years

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2.1 Abstract

Long-chain polyunsaturated fatty acid (LCPUFA) supplementation of formula can have beneficial effects on neurodevelopmental outcome in early infancy, but uncertainty exists regarding effects after 6 months. The current study is the first to investigate whether consumption by term infants of formula containing LCPUFA for the first two months after birth improves neurological condition of these children at 9 years of age. A prospective, double-blind, randomized control study was performed in two groups of healthy term infants: a control group with standard formula (CF, n169) and a LCPUFA-supplemented group (LF, n146). A breastfed group (BF; n159) served as a reference. At 9 years, children were neurologically assessed according to Touwen, resulting in a Neurological Optimality Score (NOS) and information on severity and type of minor neurological dysfunction (MND). Information on potential confounders was collected at enrolment and follow-up. Multivariate analyses were carried out to evaluate the effect of nutrition while adjusting for confounders. Attrition (28%) was selective: dropouts in the LF group were more often boys and had a significantly lower mental developmental index at 18 months. Neurological optimality and severity and type of MND at 9 years did not differ between the two formula groups. Children in the BF group showed significantly less often fine manipulative dysfunction than formula-fed children. In conclusion, LCPUFA supplementation of formula during the first two postnatal months in healthy term infants does not alter neurological function at school age. The study confirmed that breastfed infants have a slightly better neurodevelopmental outcome than formula-fed infants.

2.2 Introduction

Long-Chain Poly Unsaturated Fatty Acids (LCPUFA) have become a major focus of attention in the field of infant nutrition and development. The Cochrane reviews of the group of Simmer (1,2) indicated that supplementation of formula with LCPUFA, in particular docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA; 20:4n-6), in general does not affect motor, visual and cognitive outcome. However, when the age at which the child is assessed is taken into account the results are less straightforward. Outcome of LCPUFA supplemented formula groups (especially DHA supplementation) is better in early infancy, but studies which assessed outcome between 6 and 24 months usually did not demonstrate differences between supplemented and non-supplemented groups (1). The latter age period is, however, known for its insensitivity to reveal adverse effects of exposures during early ontogeny (3). Thus, the absence of an effect between 6 and 24 months does not preclude an effect at later age. Currently data on the effect of LCPUFA supplementation of formula on neurodevelopmental outcome at school-age are lacking.

The present study aims at investigating whether consumption by term infants of formula containing LCPUFA for the first two months after birth improves the neurological condition of these children at 9 years of age. Neurological development is one of the domains in which the early beneficial effects of LCPUFA manifested itself. The study is an extension of the original Groningen LCPUFA-study, a randomized controlled trial on the effect of supplementation of formula with LCPUFA during the first two postnatal months. At the age of 9 years a standardised and age-specific neurological examination was carried out, which specifically assesses Minor Neurological Dysfunction (MND). MND is considered a valid measure of neurological condition that shows a strong relationship with pre- and perinatal adversities (3). Earlier Lanting et al (4) reported that breastfeeding was associated with less MND at school age than formula feeding. The primary outcome measure of the current study was the Neurological Optimality Score (NOS). The NOS uses the optimality concept to summarize neurological condition; it provides a sensitive measure of the child's overall neurological status. Secondary outcome measures were clinical neurological condition in terms of severity and type of MND. Two major forms of MND can be distinguished, simple and complex MND. Simple MND can be regarded as a typical but non-optimal form of brain function, whereas complex MND is the clinically relevant form of MND.

Based on the literature, we hypothesize that the LCPUFA supplemented children perform better than the children who received standard formula and that breastfed children perform better than the formula fed children. A better performance will be reflected in a higher NOS, less and less severe MND, and a lower prevalence of specific neurological dysfunctions. The effect of gender on relationships between postnatal supplementation and neurological outcome was also determined as previous studies suggested the possibility of an advantage of LCPUFA supplementation in males (5).

2.3 Methods

2.3.1 Participants

Details of the study design have been described previously(6). Mothers of 314 infants chose to bottle feed their child and 160 opted for breastfeeding. The infants receiving formula were randomized into a standard formula group (control formula, CF, n169) and a LCPUFA supplemented formula group (LF, n145). Standard formula consisted of Nutrilon Premium®. For the supplemented formula, the lipid fraction of Nutrilon Premium® was enriched with 0.45% (by wt) AA and 0.30% (by wt) DHA. The duration of supplementation was 2 months. In case breastfeeding stopped prior to 2 months, the infant received LCP-supplemented formula till the age of 2 months and, remained in the BF group. All formula-fed infants received control formula between 2 and 6 months. The children underwent neurodevelopmental assessment at 3 and 18 months of age (6,7). Follow-up was achieved in 84% (3 months (6)) and 92% (18 months (7)) of the riginal groups. All children seen at the 18 months follow-up were eligible for re-testing at 9 years. At the 9 year follow-up, both parents and examiners were unaware of the type of formula-feeding the infant had received. The examiners also were blind to the type of milk fed during the first eight weeks.

2.3.2 Procedures

Neurological condition of the children was evaluated with the examination according to Touwen (1979) (8), which is a standardised, age specific assessment designed for the assessment of minor neurological dysfunction (MND). Essential in the diagnostics of MND is the presence of coherent clusters of signs. Single signs do not have clinical significance, signs only have significance when they co-occur (cluster) with other signs within a functional domain. The examination is organized into eight functional domains: posture and muscle tone, reflexes, dyskinesia, coordination, fine manipulative ability, associated movements, sensory deficits and cranial nerve functioning. The examination results in a clinical classification: normal, simple MND, complex MND or abnormal. A child is considered neurologically abnormal in the presence of a clear neurological disorder such as cerebral palsy. Simple MND denotes the presence of one or two domains of dysfunction and is present in about 15–20% of children. It has little clinical relevance and can be regarded as typical but non-optimal brain functioning, in other words as a minor neurological difference. Complex MND denotes the presence of more than two domains of dysfunction and is the clinically relevant form of MND. In an aetiological sense it can be considered a bor-

derline form of cerebral palsy as it is linked to a chain of pre- and perinatal adversities (3,9). A child is classified as neurologically normal when no domains are scored as deviant or in case of the isolated presence of a mild dysfunction in reflex activity.

The neurological examination according to Touwen has a good intra-rater, inter-rater and test-retest reliability, the kappa statistics of the three forms of reliability for neurological classification ranged between 0.71 and 0.83 (9). Its construct validity is reflected by the differential relationship for simple and complex MND with prenatal and perinatal adversities: adverse conditions during early life have a weak to moderate relationship with simple MND and a strong correlation with complex MND (3,10). Predictive validity is good; this is reflected by the relation between the severity of MND at 9 years and the risk of MND at 12 and 14 years and that of learning and behavioural problems at 9 and 14 years (3,11,12).

The study's primary outcome parameter was the Neurological Optimality Score (NOS). The NOS uses the optimality concept to summarize neurological condition and provides a sensitive measure of the child's overall neurological status. The sensitivity of the NOS to detect effects of early nutrition may be illustrated by the study of Bouwstra et al.(13) which demonstrated that prenatal fatty acid status was related to NOS at 18 months, but not to outcome measured with the Bayley Scales of Infant Development (BSID). For 64 items, representing the entire neurological examination, an optimal range was identified (see online supplemental Table 1). The total number of items with a value within the predefined optimal range formed the neurological optimality score of a child. It should be realized that there is a conceptual difference between normality and optimality, as the range for optimal behaviour is narrower than that of normal behaviour (14). The NOS may be regarded as a quantitative and more subtle expression of the clinical neurological condition. This also means that the two measures are highly correlated (15) (current study rho = -0.691; $p < 0.001$). The quantitative and precise nature of the NOS makes it a suitable instrument to evaluate subtle deviations in neurodevelopmental outcome. The NOS has previously been used in infancy and pre-school age; in the present study NOS principles are applied for the first time at the age of 9 years.

Data on pre- and perinatal conditions had been collected during enrolment with the help of the Obstetrical Optimality Score (OOS).The OOS describes the obstetrical conditions ranging from the parents' socio-economic status to the infant's condition immediately after birth (15). At the assessment of 18 months maternal verbal intelligence (IQ) was estimated using a very abbreviated version of the Wechsler Adult Intelligence Scale (WAIS III), limited to the subtests information and vocabulary (16). Social condition was documented with the Home Observation for Measurement of the Environment (HOME) inventory (17). The HOME contains 45 items clustered into 6 subscales: Parental Responsivity, Acceptance of Child, Organization of the Environment, Learning Materials, Parental Involvement, and Variety in Experience. At the 9 year follow-up information was collected on parental education and profession, the child's medical history, family composition and nutritional habits.

Depending on the wish of the participants, the assessment was carried out in the hospital or at home. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Groningen University Hospital. Written informed consent was obtained from all subjects. The trial is registered under ISRCTN52788665.

2.3.3 Statistical analysis

Statistical analysis focused on differences in neurological outcome between the two randomized formula groups. In addition differences between the breastfed group and the formula groups were analysed. Specific attention was paid to the effect of site of investigation and maternal estimated verbal IQ. Neurological classification, specific clusters of dysfunction and NOS were not normally distributed. Univariate analyses were performed with the Mann-Whitney U test and the Kruskall-Wallis test.

In order to analyse the effect of nutritional group on neurological classification and type of dysfunction while taking into account the role of potential confounders logistic regression analyses were performed. The following confounders, associated with neurological outcome at $p < 0.20$, were entered into the analyses: gender, maternal smoking during pregnancy, duration of the second stage of delivery, birthweight, OOS, maternal hypertension during pregnancy, Apgar score 3 minutes after birth, and maternal educational level. To investigate the effect of nutritional group and the above mentioned confounders on NOS multiple linear regression was applied to the NOS transformed to the fifth power. This transformation to normality was identified by the Box-Cox method applied to regression model residuals (the NOS was skewed to the left).

The multivariate analyses on the effect of nutritional group were carried out in two steps. In the first analysis the effect of the type of formula was assessed, in the second analysis the difference between formula and breastfeeding was evaluated. A p-value 0.05 or less was considered statistically significant. Statistical analyses were performed using SPSS 14.0 for Windows (SPSS, Inc, Chicago, IL).

2.4 Results

All children who were tested at 18 months were invited for follow-up at 9 years (n436); 341 of the 474 children of the original study agreed to participate, 91 children in the LF-group (63%), 123 children in the CF-group (73%) and 127 children in the BF-group (79%; Figure 1). Obstetrical data of the study groups and sociodemographic characteristics of the parents are described in Table 1.

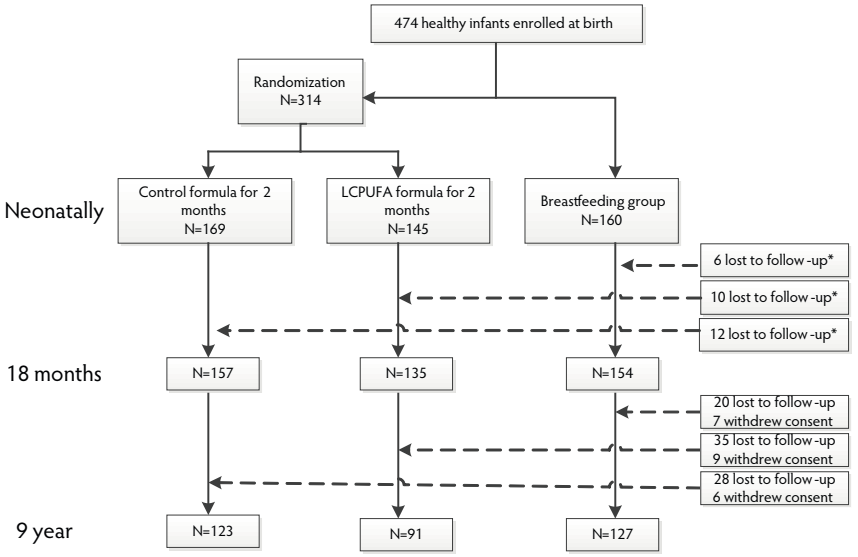

Figure 1. Flow diagram of children enrolled in the study and followed up until 9 years of age.
* for more detailed information see (7).

In general, obstetrical and social characteristics of the children who were and who were not assessed at 9 years were comparable. Yet, children who were not assessed at 9 years had shown significantly more often normal-optimal general movements at 3 months than those who had been assessed, meaning that children with an optimal neuromotor condition in early infancy were underrepresented in the follow-up of the three groups at 9 years ($p = 0.003$). In addition, attrition in the LF group was more selective than in the other groups. First, in the LF-group more boys did not take part in the follow-up (35 and 17 girls) compared to the BF-group (15 boys and 17 girls) and the CF-group (24 boys and 17 girls). Second, the mental developmental index of the Bayley Scales of Infant Development (18,19) at 18 months of the children of the LF group who were not assessed at 9 years was significantly lower than that of LF-children who did participate in the follow-up at 9 ($p = 0.007$). A similar selective attrition was not present in the CF and BF groups.

Table 1. *Obstetrical and social characteristics of the three groups assessed at 9 years.*

Variable	BF group (*n*127)	LF group (*n*91)	CF group (*n*123)
Retention (% of original study groups)	79%	63%	73%
Gender: boys/girls	64/63	42/49	71/52
Duration second stage of the delivery (minutes), median (range)	25 (2–138)	22 (1–120)	22 (1–146)
Birth weight (g), mean (SD)	3588 (436)	3527 (498)	3518 (473)
Apgar score 3 minutes after birth, median (range)	10 (5–10)	10 (7–10)	10 (7–10)
Maternal education *			
–high (university education or vocational college), n (%)	60 (49%)	13 (15%)	15 (13%)
–medium (college graduate or junior vocational college), n (%)	57 (46%)	52 (60%)	85 (72%)
–low (no education or primary education), n (%)	6 (5%)	22 (25%)	18 (15%)
Presence of maternal smoking during pregnancy, n (%) #	25 (20%)	31 (34%)	44 (36%)
Presence of maternal hypertension during pregnancy, n (%)	11 (9%)	11 (12%)	21 (17%)
HOME, median (range)	44 (39–45)	43 (32–45)	43 (35–45)
OOS, median (range)	60 (43–69)	59 (50–67)	59 (49–67)

* significant difference between all groups p = 0.001; # significant difference between BF and LF/CF combined p = 0.003.

OOS = Obstetrical Optimality Score; HOME = Home Observation for the Measurement of the Environment (HOME) inventory; BF = Breastfed group; LF = LCPUFA supplemented group; CF = Control group.

Neurological condition at 9 years was not affected by the site of investigation (in the hospital or at home), by current consumption of fish (dichotomized as at least once a week or less than once a week) and maternal verbal IQ. The NOS of children in the LF and CF groups did not differ, in both groups the median NOS score was 57. The NOS of formula-fed children (LF and CF groups) was significantly lower than that of breastfed children (median values 57 and 58 respectively, Mann-Whitney, p = 0.008; see also Table 2 for more specifics on the BF-group).

Table 2. *Duration of exclusive breastfeeding and Neurological Optimality Score*

Duration of exclusive breastfeeding	3–5 weeks	6–8 weeks	> 8 weeks
Number of children	25	23	62
NOS (median (range))	58 (51–63)	58 (50–63)	58 (47–64)

Multiple regression (Table 3) confirmed that the NOS was not affected by LCPUFA supplementation. The analysis revealed an interaction between gender and type of feeding: girls who had been breastfed had a higher NOS than girls who had been formula fed. Other factors associated with a higher NOS were higher birthweight and an OOS above the 10th percentile.

Table 3. *Results of linear regression analysis of factors contributing to the normalized NOS (the fifth power of NOS/50).*

Variables		Effect	P-value	95%-Confidence interval
Type of feeding within gender				
males	LF vs CF (reference)	0.173	0.137	−0.055 , 0 401
	BF vs CF	0.141	0.162	−0.057 , 0.340
	BF vs LF	−0.031	0.788	−0.261 , 0.198
females	LF vs CF	−0.100	0.389	−0.328 , 0.128
	BF vs CF	0.238	0.029	0.024 , 0.452
	BF vs LF	0.338	0.002	0.122 , 0.555
Gender within type of feeding				
CF	female vs male	0.367	0.001	0.156 , 0.578
BF	female vs male	0.464	0.001	0.262 , 0.665
LF	female vs male	0.094	0.450	−0.150 , 0.338
Birthweight (kg)		0.147	0.032	0.012 , 0.282
OOS: above vs below the 10th percentile		0.211	0.075	−0.021 , 0.443

n330; $R^2 = 0.14$; intercept for a CF male with birthweight 3.5 kg and OOS above P10: 1.7265; BF / LF / CF: Breastfed / LCPUFA supplemented / Control group; OOS = Obstetrical Optimality Score

The effects presented in Table 3 are on the transformed scale of NOS. An effect of size, for example, 0.4 on the transformed scale can be interpreted on the original scale as follows. A NOS value of 57 transforms to (57/50)5 = 1.9254, increasing this by 0.4 gives 2.3254, transforming this back to the original scale gives $50 \times 2.32540.2 = 59.2$. Note that as the width of most confidence intervals in Table 3 is below 0.4, the CI width thus becomes about 2 points on the original NOS scale. The NOS at 9 years showed a statistically significant association with the mental developmental index of the Bayley Scales of Infant Development at 18 months (Spearman rho = 0.193, $p < 0.0001$).

Forty six to 54% of children had a normal neurological condition, 36–39% showed simple MND and 10-15% of children had complex MND. Neurological classification did not differ between the randomized formula groups. Children in the BF group tended to have a slightly better neurological condition than the children in the formula fed groups, but the difference did not reach statistical significance (p = 0.17; Table 4).

Table 4. *Neurological classification per nutritional group.*

	BF group n = 127	**LF group n = 91**	**CF group n = 123**
Neurologically normal	68 (54%)	44 (48%)	56 (46%)
Simple MND	46 (36%)	35 (39%)	48 (39%)
Complex MND	13 (10%)	12 (13%)	19 (15%)

No statistically significant differences were present between the groups

BF = Breastfed group; LF = LCPUFA supplemented group; CF = Control group

LF and CF groups did not differ in the prevalence of specific types of dysfunction. Children of the BF group however, showed significantly less often fine manipulative dysfunction than formula fed children (BF:16%, LF 30%, CF31%; BF versus formula groups, p = 0.002), this was confirmed in multivariate analysis (Table 5) with the following contributing factors: OOS below the 10th percentile, male gender, maternal smoking during pregnancy and the duration of the second stage of delivery. Breastfed and formula fed children did not differ in the prevalence of other types of neurological dysfunction.

Table 5. *Results of logistic regression analysis of factors contributing to fine manipulative dysfunction (explained variance of 18.5%)*

Contributing factors	**OR**	**P**	**95%–Confidence interval**
Type of feeding:			
BF (reference)	1		
LF	2.668	0.011	1.3 , 5.7
CF	2.548	0.009	1.3 , 5.2
Covariates:			
Male gender	2.151	0.010	1.2 , 3.9
OOS below the 10th percentile	7.271	0.001	2.3 , 23
Maternal smoking	1.935	0.034	1.1 , 3.6
Duration of the 2nd part of the delivery	0.989	0.032	0.98 , 1

BF = Breastfed group; LF = LCPUFA supplemented group; CF = Control group;

OR-Odds Ratio; CI = Confidence Interval; OOS = Obstetrical Optimality Score

2.5 Discussion

The present study indicated that consumption by term infants of formula containing LCPUFA for the first two months after birth did not affect neurological condition at 9 years. The study also revealed that fine manipulative ability of breastfed children was better than that of formula fed children.

A major limitation of the study is its attrition. Overall attrition was 28%, which – over a period of 9 years – can be regarded as relatively favourable (20). However, a major problem in the current study was the selective nature of attrition, that is, a selective loss of boys and children with a worse cognitive development at 18 months in the LF group. The selective attrition interfered with the randomized design of the study. While the multiple regression analysis can alleviate the effect of attrition if the missingness of data depends only on known covariates, it cannot annihilate the effect of severe ("missing not at random") attrition.

Some of the breastfed children received a few weeks formula supplemented with LCPUFA (Table 2). This may be considered a limitation as it may have influenced any differences between the supplemented formula group and the breastfed group. However, the finding that the neurological condition of the LF-group did not differ from the CF-group, reduces the likelihood that differences between LF-group and BF-group were affected by the mixed composition of the BF-group.

The prevalence of MND in the present study is another point which deserves methodological attention. The prevalence is higher than indicated by earlier estimations of MND in the general population (3). This may raise questions regarding the representativeness of the current sample of healthy full-term infants. However, neurological evaluation of these groups at 3 months indicated that neurological status was representative for the general population (6,21). Possibly, the relatively high prevalence of MND in the present study reflects the general trend of worsening neuromotor condition in the last decades (22).

The strengths of the study are its randomized design and its assessor-blinded evaluation with an internationally recognized, sensitive technique to evaluate neurological condition (4,23-26). Based on the width of the confidence intervals, it is concluded that the LF versus CF differences do not exceed 2.2 points on the NOS scale.

This is the first study reporting the effect of supplementation of formula with LCPUFA in healthy term infants on neurological condition at school age. Using the NOS, a sensitive indicator of neurological condition, an effect of 2 months postnatal LCPUFA supplementation on neurological status at 9 years could not be demonstrated. Bearing in mind the fact that the LF group suffered from selective attrition of children with a lower mental developmental index at 18 months, the current study indicates that LCPUFA supplementation during the first two postnatal months does not promote neurological condition at school age.

The current finding of a subtle positive association between breastfeeding and neurodevelopmental outcome, and neurological condition in particular, is in line with reports of others (4,27,28). The association between breastfeeding and neurological outcome was less strong than previously reported by Lanting et al. (1994) (4), which may be attributed to differences in the populations studied (Lanting et al: a mix of high risk and low risk infants; current study: healthy full term infants) and the quality of the breastfeeding data (Lanting et al: retrospectively collected information; current study: detailed prospective information). Der et al.(29) was able to demonstrate that maternal IQ accounted for major part of the association between breastfeeding and developmental outcome in terms of IQ. In the current study, a subtle association between breastfeeding and fine manipulative ability remained, also when estimated maternal verbal IQ was taken into account. Fine manipulative ability is mediated especially by cortical-subcortical networks. These networks do not only play a role in sensorimotor aspects of motor programming, movement planning, program selection and motor memory but also in cognitive functions, such as intelligence (30,31). This means that the association between breastfeeding and better fine manipulative ability corresponds to reports of others of an association between breastfeeding and higher IQ (28). Interestingly, the NOS data indicated that especially girls profit from the beneficial effect of breastfeeding. This gender specific effect may be explained by shared genome, and, albeit less likely, gender-specific differences in metabolism of α-linolenic acid (ALA): adult women have a higher conversion rate of ALA to eicosapentaenoic acid and DHA than adult men (32).

In conclusion, the current study indicates that LCPUFA supplementation of formula during the first two postnatal months in healthy term infants does not promote neurological condition at school age. In addition, the study confirmed that breastfed infants have a slightly better neurodevelopmental outcome than formula-fed infants – reflected in the present study by a reduced prevalence of fine manipulative dysfunction. Finally, the study underscores the need of the evaluation of selective attrition with respect to early developmental data in studies assessing the effect of early nutrition on long term developmental outcome.

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2.6 Online supplemental Table 1

Neurological Optimality Score of the GOM in children aged 9 years

Items	Criteria
Cluster Posture and Muscle Tone	
– sitting, standing and walking	– ability to perform independently
– posture while sitting	– typical posture of head, trunk, arms and legs
– extending arms in pronation and supination while sitting	– ability to stabilize the arms in space
– voluntary relaxation	– easy
– muscle strength in head, trunk, arms and legs	– age-adequate strength
– muscle tone of the head and trunk	– normal muscle tone
– muscle tone of the arms and legs	– normal muscle tone
– range of the head, trunk, arms and legs	– typical range
– posture while standing	– typical posture of the head, trunk, arms and legs
– posture while walking	– typical posture of the head, trunk, arms and legs
– walking on toes and on heels	– able to walk on toes and on heels
Cluster Reflexes	
– reflex threshold of the biceps muscle, triceps muscle, quadriceps muscle and Achilles tendon	– normal reflex threshold
– reflex intensity of the biceps muscle, triceps muscle, quadriceps muscle and Achilles tendon	– normal reflex intensity
– foot sole response	– plantar flexion or no response, symmetry
– plantar grasp	– bilaterally absent
– abdominal skin reflex	– symmetrically present
Cluster Involuntary Movements	
– presence of choreiform movements during test of extended arms in sitting	– no
– presence of athetotiform movements during test of extended arms in sitting	– no
– presence of tremor movements during test of extended arms in sitting	– no
– Test of involuntary movements while standing: distal choreiform movements	– no
– Test of involuntary movements while standing: proximal choreiform movements	– no
– Test of involuntary movements while standing: athetotiform movements	– no
– Test of involuntary movements while standing: tremor	– no
– Choreiform movements of the eyes during fixation and pursuit	– no
– Choreiform movements of the face during fixation and pursuit	– no
– Presence of choreiform movements during sticking out the tongue	– no

Items	Criteria
Cluster Coordination Problems	
– kicking with the lower leg while sitting	– age-adequate performance
– being pushed while sitting	– age-adequate ability to keep balance
– being pushed while standing	– age-adequate ability to keep balance
– Romberg test	– ability to stand still or only move toes
– diadochokinesis	– age-adequate performance
– finger-nose test	– age-adequate performance
– fingertip-touching test	– age-adequate performance
– walking on a straight line	– age-adequate ability to keep balance
– standing on one leg	– ability to maintain balance for 20 seconds on each leg
– hopping on one leg	– ability to hop 20 consecutive times on each leg
– knee-heel test	– correct placing and straight sliding of both legs
Cluster Fine Manipulative Ability	
– finger opposition test, smoothness	– age-adequate
– finger opposition test, transition	– age-adequate
– circle test, opposite direction	– age-adequate
– circle test, same direction	– age-adequate
– circle test, transition	– age-adequate
– follow a finger test	– age-adequate
Cluster Associated Movements	
– mouth-opening-finger-spreading phenomenon	– age-adequate
– diadochokinesis	– age-adequate
– finger opposition test	– age-adequate
– walking on toes	– age-adequate
– walking on heels	– age-adequate
Cluster Sensory Deficits	
– graphesthesia	– typical
– kinesthesia	– typical
– sense of position	– typical
– vision, need of spectacles	– no need
– hearing	– typical
Cluster Cranial Nerve Functioning	
– facial motility	– normal motility
– position of the eyes	– absence of strabismus, heterophoria or other abnormalities of the position of the eyes
– fixation of the eyes	– normal
– pupil reaction	– normal pupillary reactions
– pursuit movements of the eyes	– smooth movements
– nystagmus	– absent
– tongue motility	– typical motility
– speech	– typical speech
– pharyngeal arches	– normal symmetrical reaction
– visual fields	– typical size
Quality of walking	
– gait width, gait quality and heel-toe gait	– normal gait width, gait quality and heel-toe gait

Chapter 3

Effects of long-chain polyunsaturated fatty acids supplementation of infant formula on cognition and behaviour at 9 years of age

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3.1 Abstract

Aim: Long-chain polyunsaturated fatty acid (LCPUFA) formula supplementation may be beneficial for cognitive development. This study aimed to investigate the effect of LCPUFA formula supplementation, primarily on cognition and secondarily on behaviour at 9. Special attention was paid to a potentially modifying effect of maternal smoking during pregnancy.

Method: A double-blind, randomized control study was performed in healthy term infants: a standard formula control group (CF, n = 169) and a LCPUFA-supplemented group (LF, n = 146). A breastfed group (BF; n = 159) served as an additional reference. At 9 years, 72% of children (52% male) underwent extensive cognitive and behavioural testing.

Results: An interaction between infant nutrition and smoking during pregnancy was found. In children exposed to smoking during pregnancy LF was associated with higher mean scores on verbal IQ ($p = 0.007$) and learning and memory ($p = 0.006$), in children not exposed to smoking during pregnancy LF was associated with lower mean scores on verbal memory ($p = 0.003$). The LF group scored worse than the CF group on executive function ($p = 0.001$). Breastfeeding was associated with better performance on IQ ($p = 0.005$).

Interpretation: No consistent beneficial effect of LCPUFA formula supplementation on cognitive development in term infants was found. The study confirmed that breastfeeding is associated with better cognition.

3.2 Introduction

Long-term developmental effects of supplementing formula with long-chain polyunsaturated fatty acids (LCPUFA) remain a matter of debate. LCPUFA are essential, the most important are docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA; 20:4n-6). They are present in breastmilk, but a decade ago not in standard infant formulae.

Young infants have a high DHA and AA need and a limited capacity to synthesize these fatty acids,(1) reflected by declining LCPUFA levels in plasma or red blood cell membranes when fed non-supplemented formulae. Current recommendations indicate that infant formulae should contain LCPUFA in quantities comparable to breastmilk.(2)

Several studies demonstrated that breastfed infants have a better cognitive outcome than infants fed formula.(3) However, differences between breastfed and formula fed children can not only be attributed to nutrition, but are also explained by related factors, such as higher maternal IQ(3) and genotype.(4)

Interestingly, Batstra et al.(5) reported that breastfeeding abolished the negative effect of exposure to maternal smoking during pregnancy on cognitive outcome at school age. Exposure to maternal smoking during pregnancy is known to be associated with a negative effect on the child's behavioural and cognitive outcome.(6) Possibly this negative effect is mediated by an adverse effect of maternal smoking on the infant's capacity to synthesize AA and DHA,(7) which in turn could lead to a subclinical LCPUFA deficiency at birth.

Systematic reviews concluded that LCPUFA supplementation is associated with improved developmental outcome during early infancy. However, no benefits of supplementation were found when follow-up took place between 6 and 24 months of age.(8) Studies reporting cognitive and behavioural follow-up at school age are lacking.

The current study is part of the Groningen LCPUFA study, a randomized controlled trial on the effect of formula supplementation with LCPUFA during the first two postnatal months in healthy term infants. Previously the study reported that LCPUFA supplementation and breastfeeding were associated with a developmental advantage at 3 months but not at 18 months.(9,10) Recently the participants of the study were re-assessed at 9. The reassessment indicated that LCPUFA supplementation did not affect the presence or absence of minor neurological dysfunction and that breastfed children showed fine manipulative disability less often than formula fed infants.(11) In the present report we focus on cognitive abilities and behaviour.

Studies in infant baboons showed that LCPUFA are incorporated in the brain's gray matter, in particular the cerebral cortex and the basal ganglia.(12) Consequently, it is likely that functions such as motor learning, intelligence, executive functions, attention, and memory which heavily depend on distributed neuronal networks including cortical areas and basal ganglia, will benefit most.(13) Therefore we hypothesize that LCPUFA supplemented children will score higher than children fed control formula on tests of intelligence, executive function, attention and learning and memory. Also, we hypothesize that breast-

fed children perform better on cognitive tests than formula fed children. More specific, we expect that a potential beneficial effect of LCPUFA is reflected especially in improved intelligence, executive function, attention, learning and memory, and less in an effect on language ability. Both cognition and behaviour are the result of a continuous interaction between genetic background and environment. However, behaviour is more strongly affected by environmental conditions such as family functioning than cognition.(14,15) Therefore we consider behaviour as a secondary outcome, which is not or minimally only affected by LCPUFA supplementation.

3.3 Methods

3.3.1 Participants

The study is an extension of a double-blind randomized controlled trial investigating the effect of LCPUFA-supplementation on development of healthy term infants. The study was designed to detect a difference of 7.5 on the mental developmental index of the Bayley test at 18 months between the LF and CF groups. Fifty-seven children had to be included in each group (for details see (9)). In parallel a group of breast-fed healthy term infants was enrolled which served as a reference group. Pregnant women were recruited between 1997 and 1999. Mothers of 312 infants chose to bottle feed and 160 opted for breastfeeding. The infants receiving formula were randomized into standard formula group (control formula, CF, n = 169) or LCPUFA supplemented formula group (LF, n = 145) by means of a single central computerized randomization that used a block design (blocks of 6 delivered in batches of 78). Standard formula was Nutrilon Premium®. For the supplemented formula, the lipid fraction of Nutrilon Premium® was enriched with 0.45% (by wt) AA and 0.30% (by wt) DHA. Supplementation duration was 2 months. When breastfeeding stopped before 2 months, the infant received LCPUFA-supplemented formula till 2 months. Fifty percent of mother stopped breastfeeding when the infant aged 2 months, which conforms to Dutch breastfeeding practices.(16) All formula-fed (FF) infants received control formula between 2 and 6 months. All 472 participating infants were born at 37-42 week of gestation and were of native western European origin. Excluded were infants with a congenital disorder interfering with adequate daily functioning, twins, infants whose mothers did not have mastery of the Dutch language or who suffered from significant illnesses or disability, adopted and foster infants and formula fed infants who had received human milk for more than 5 days.

Children assessed at 18 months (436, 92% of the original 459) were invited at 9 years. Parents and examiners were blind to type of formula-feeding the infant had received. The examiners were also blind to formula versus breast status.

3.3.2 Procedures

The assessment at 9 consisted of intelligence testing, evaluation of cognitive functions, neurological evaluation in terms of minor neurological dysfunction, behavioural evaluation, blood pressure measurements and anthropometrics. In the current report intelligence, cognition and behaviour are addressed. Intelligence was assessed using the WASI – Wechsler Abbreviated Scale of Intelligence.(17) This resulted in a Full IQ (FIQ) score, made up of a Verbal IQ (VIQ) score and a Performance IQ (PIQ) score. The WASI is a valid screening instrument.(18)

Cognitive performance was assessed with the NEPSY, Tea-Ch and Children's Memory Scale. The NEPSY(19) is designed to assess neuropsychological functioning in 3 to 12 year old children. Three NEPSY-domains were evaluated: 1) Attention and Executive Functions (Tower test – further denoted as executive function), 2) Memory and Learning ability (Memory for Names, Memory for Faces and Narrative Memory) and 3) Language ability (Comprehension of instructions and Speeded naming). The latter domain was included to serve as a control domain in which we did not expect a nutritional effect. The Tea-Ch – Test of Everyday Attention for Children – is a standardized and valid test for the assessment of attention in children aged 6 to 16 years.(20) All domains were assessed, however using a reduced set of subtests: 1) Selective attention (using the Sky Search test), 2) Sustained attention (using the Score test and Sky Search DT test) and 3) Attentional control/switching (using the Creature Counting test and Opposite Worlds test; note that switch attention is also known as divided attention). Also verbal memory was assessed with the word Pair subtask of the Children's Memory Scale. For all tests the original norms were used as Dutch norms were not available.

Behaviour was evaluated by means of parental and teacher's questionnaires, i.e., the Dutch versions of the Children's Behavioural Check List (CBCL)(21) and the Teacher Report Form (TRF).(21) The reliability and validity of CBCL and TRF are well established.(21,22)

Data on pre- and perinatal conditions were collected during enrolment using the Obstetrical Optimality Score (OOS).(23) At the 18 months assessment maternal verbal intelligence (IQ) was estimated with an abbreviated version of the Wechsler Adult Intelligence Scale (WAIS III), using the subtests information and vocabulary.(24) At the 9 year follow-up, information was collected on parental education and profession, the child's medical history, nutritional habits and family composition.

The neuropsychological assessments were carried out by two psychologists, depending on the wish of the parent either at the hospital or at the home of the children. The study was conducted according to the guidelines laid down in the Declaration of Helsinki all procedures involving human subjects were approved by the Ethics Committee of the Groningen University Hospital. Written informed consent was obtained from all participants. The trial is registered under ISRCTN52788665.

3.3.3 Statistical analysis

The purpose of the statistical analyses was to compare the two randomized formula feeding groups and the breastfeeding group with respect to ten cognitive outcome variables and secondarily to behavioural variables while adjusting for possible confounders. As IQ and specific cognitive variables appeared to be normally distributed we used multiple linear regression analysis for these 10 outcomes. The behavioural outcomes, that were not normally distributed, were dichotomized into normal versus borderline/clinical according to Dutch norms,(21) and analysed by logistic regression analysis.

We included the following variables as potential confounders: gender, maternal estimated verbal IQ, maternal level of education (1 = low, 2 = medium, 3 = high), duration of breast feeding and pre-pregnancy maternal body mass index (BMI). Special attention was paid to smoking during pregnancy. In line with other developmental studies, smoking was dichotomized into no/minimal smoking defined as <5 cigarettes per day and evident smoking defined as ≥5 cigarettes per day.(7) In the regression analysis we also explored the existence of interactions between the feeding type and the potential confounders whose main effects were significant at 5% level. The results are presented as either differences between adjusted means of the three feeding groups or as adjusted odds ratios, with the corresponding 95%-confidence intervals (CI) and P-values.

The CI's and P-values are not corrected for multiple testing. To (Bonferroni-) correct the three pairwise comparisons between feeding groups the P-values could be multiplied by three. This is however not appropriate if one is primarily interested in comparing the two randomized formula groups. To correct for the multiplicity of tests for interactions is more complicated. As a rough solution we have chosen to test them at 1% level. As the analysis involved 10 outcome variables, a Bonferroni correction for multiplicity can be contemplated as well. It is up to the reader to decide for which group of cognitive tests this should be applied. The analyses were performed using SPSS 14.0 for Windows (SPSS, INC, Chicago IL).

3.4 Results

Of the 436 eligible children 341 participated (72% of the original study group n = 474, Figure 1) Obstetric and sociodemographic characteristics are described in Table 1. In general, obstetric and social characteristics of the children who were and who were not assessed at 9 years were comparable, including maternal smoking during pregnancy. Yet the children who took part in the follow-up at 9 years showed a less optimal neuromotor condition in early infancy. Also, in the LF group a selective attrition of boys (LF: 36 male 18 female,

CF: 27 male 19 female, BF: 15 male 18 female; p = 0.003) and of children with a lower mental developmental index (MDI) at 18 months (MDI of children not assessed at 9 years: LF = 112, CF = 118, BF = 121; p = 0.007) had occurred.

None of the test results were affected by current consumption of fish or the duration of exclusive breastfeeding.

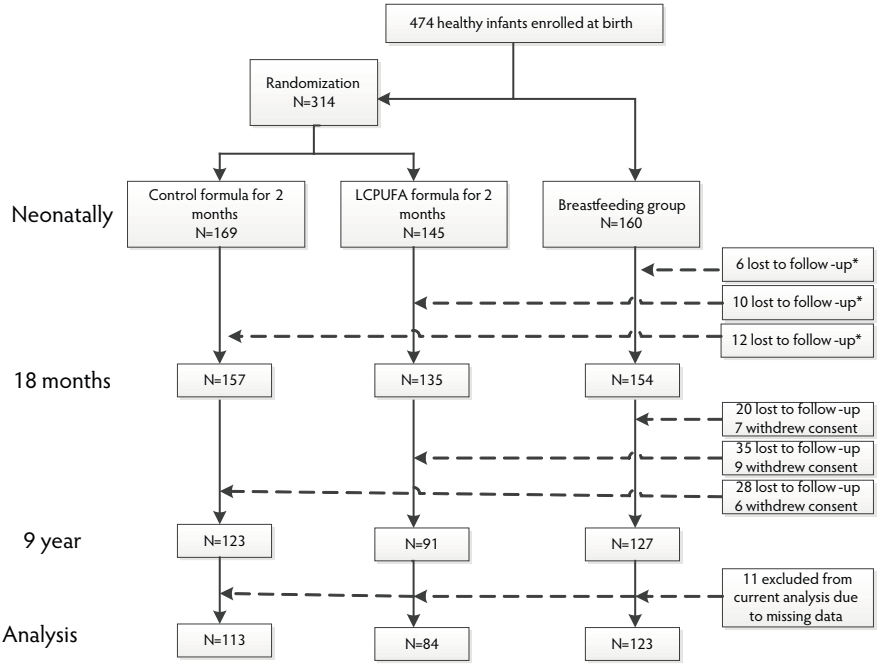

Figure 1. Flow diagram of children enrolled in the study and followed up until 9 years of age.
* for more detailed information see (10)

Table 1. *Obstetric and social characteristics of the three groups assessed at 9 years*

Variable	CF group (n = 123)	LF group (n = 91)	BF group (n = 127)
Gender, boys/girls	71 / 52	42 / 49	64 / 63
Maternal obesity before pregnancy (BMI≥25), %	60%	47%	39%
Weight gain during pregnancy, median (range)	13 (0–46)	13 (0–26)	14 (0–31)
Presence of maternal smoking >5 cig/day during pregnancy, %#	23%	19%	10%
Presence of maternal hypertension during pregnancy, %	17%	12%	9%
Birth weight (g), mean (SD)	3518 (473)	3527 (498)	3588 (436)
Apgar score after 3 minutes, median (range)	10 (7–10)	10 (7–10)	10 (5–10)
OOS, median (range)	59 (46–67)	59 (50–67)	60 (43–69)
Maternal education *			
- high a, %	13%	15%	49%
- medium b, %	72%	60%	46%
- low c, %	15%	25%	5%

[a] University education or vocational college

[b] College graduate or junior vocational college

[c] No education or primary education

*significant difference between all groups p = 0.001

OOS = Obstetrical Optimality Score

3.4.1 Nutrition and intelligence

Multiple regression analysis of FIQ (Table 2) revealed an interaction between infant nutrition and smoking during pregnancy. In children of mothers who had smoked during pregnancy the mean FIQ of CF children was lower than that of BF children (Table 2a, Figure 2). Mean FIQ of LF children prenatally exposed to maternal smoking did not differ from that of the CF or BF children. In the group children whose mothers had not smoked during pregnancy nutrition did not affect FIQ.

Verbal IQ and performance IQ showed different relationships with infant nutrition. Multiple regression analysis of VIQ (Table 2a) revealed an interaction between nutritional group and smoking during pregnancy. In the children prenatally exposed to maternal smoking mean VIQ of LF children was significantly higher than that of CF children (p = 0.007; figure 3) and the mean VIQ of BF children did not differ significantly from

that of the other two groups. In the group of children whose mother did not smoke during pregnancy nutrition did not affect VIQ. The multiple regression analysis indicated that nutrition was not associated with PIQ (data not shown).

Table 2a. *Analysis of factors contributing to Full IQ and Verbal IQ.*

		Full IQ			Verbal IQ		
		Effect	95% CI	P-value	Effect	95% CI	P-value
Type of feeding within smoking during pregnancy							
Non-Smoking	LF vs CF	-3.71	-7.47 , 0.05	0.053	-3.65	-7.16 , -0.14	0.028
	BF vs CF	0.713	-2.96 , 4.38	0.703	0.54	-2.90 , 3.97	0.213
	BF vs LF	4.43	0.546 , 8.3	0.026	4.19	0.56 , 7.83	0.020
Smoking	LF vs CF	7.19	-0.60 , 14.97	0.070	9.94	2.69 , 17.19	0.007
	BF vs CF	12.21	3.68 , 20.73	0.005	10.02	2.08 , 17.96	0.014
	BF vs LF	5.02	-4.38 ;14.42	0.294	0.085	-8.67 , 8.84	0.980

n = 301; FIQ $R^2 = 0.29$; VIQ $R^2 = 0.28$

BF / LF / CF: Breastfed / LCPUFA supplemented / Control group

CI: Confidence Interval

Adjusted for: gender, maternal verbal IQ, maternal education and maternal BMI before pregnancy.

3.4.2 Nutrition and executive function and attention

In the domain of executive function and attention no interaction between smoking during pregnancy and nutrition was found. The multivariate analysis showed a lower mean performance of the LF group for executive function compared to the CF group, but no differences between the LF and BF groups (Table 2b). Infant nutrition was not associated with attention measured with the NEPSY nor with the attention parameters of the Tea-Ch.

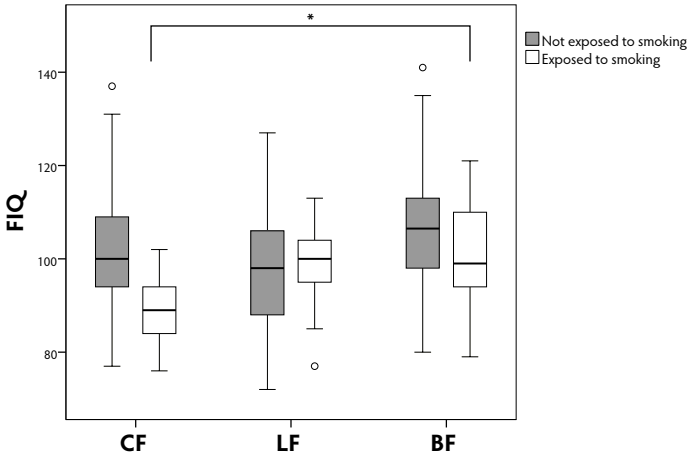

Figure 2. Full IQ: effect of early nutrition and prenatal exposure to maternal smoking Bold horizontal lines indicate median values (values provided); boxes represent interquartile ranges (IQRs); Vertical lines represent 1.5 IQR and small circles reflect outliers. * $p < 0.01$ when adjusted for confounders

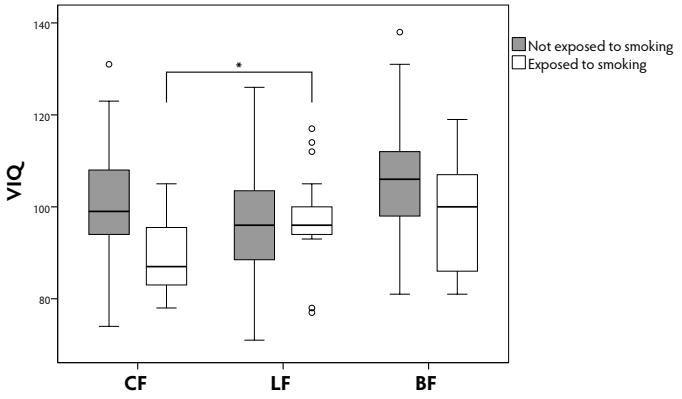

Figure 3. Verbal IQ: effect of early nutrition and prenatal exposure to maternal smoking Bold horizontal lines indicate median values (values provided); boxes represent interquartile ranges (IQRs); Vertical lines represent 1.5 IQR and small circles reflect outliers. * $p < 0.01$ when adjusted for confounders

Table 2b. *Analysis of factors contributing to the NEPSY subscale executive function*

Type of feeding	Executive function		
	Effect	95% CI	P-value
LF vs CF (ref)	-1.25	-2.01 ; -.49	0.001
BF vs CF	-.243	-1.00 ; .52	0.529
Bf vs LF	1.01	0.21 ; 1.8	0.014

n = 299; executive function $R^2 = 0.059$, intercept 10.45

CF / LF / BF: Control / LCPUFA supplemented / Breastfed group

CI: Confidence Interval

Adjusted for gender, maternal verbal IQ, maternal education, maternal smoking during pregnancy and maternal BMI before pregnancy.

3.4.3 Nutrition and learning and memory

For learning and memory multiple regression analysis revealed an interaction between infant nutrition and maternal smoking during pregnancy. In children of smoking mothers the LF children scored significantly better than the CF children whereas mean performance of BF children did not differ significantly from that of CF and LF children (Figure 4). In the group of children whose mother did not smoke during pregnancy nutrition did not affect learning and memory.

Also for verbal memory an interaction between maternal smoking and infant nutrition was found. In children whose mother smoked verbal memory was not affected by infant nutrition (Table 2c, figure 5). However, in children not exposed to maternal smoking LF children had mean lower verbal memory scores than CF children whereas no evidence of a difference was found between BF children and either of the two formula groups (Table 2c).

Table 2c. *Analysis of factors contributing to learning and memory and verbal memory*

Contributing factors:		Learning and memory			Verbal memory		
		Effect	95% CI	P-value	Effect	95% CI	P-value
Type of feeding within smoking during pregnancy							
Non-Smoking	LF vs CF	-0.617	-1.28 ; 0.04	0.066	-1.49	-2.48 ; -0.50	0.003
	BF vs CF	0.25	-0.38 ; 0.88	0.438	-0.92	-1.88 ; 0.04	0.059
	BF vs LF	0.867	0.19 ; 1.54	0.012	0.571	-0.44 ; 1.58	0.270
Smoking	LF vs CF	1.883	0.55 ; 3.22	0.006	1.17	-0.87 ; 3.21	0.260
	BF vs CF	1.64	0.16 ; 3.13	0.030	1.37	-0.88 ; 3.62	0.230
	BF vs LF	-0.24	-1.87 ; 1.39	0.770	0.206	-2.2 ; 2.6	0.870

n = 330; Learning and memory $R^2 = 0.17$; verbal memory $R^2 = 0.077$.

BF / LF / CF: Breastfed / LCPUFA supplemented / Control group

CI: Confidence Interval

Adjusted for gender, maternal verbal IQ, maternal education and maternal BMI before pregnancy.

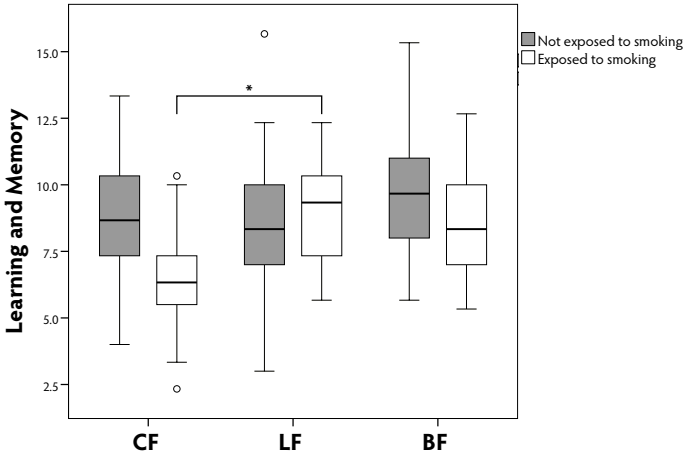

Figure 4. Learning and Memory: effect of early nutrition and prenatal exposure to maternal smoking Bold horizontal lines indicate median values (values provided); boxes represent interquartile ranges (IQRs); Vertical lines represent 1.5 IQR and small circles reflect outliers * $p < 0.01$ when adjusted for confounders

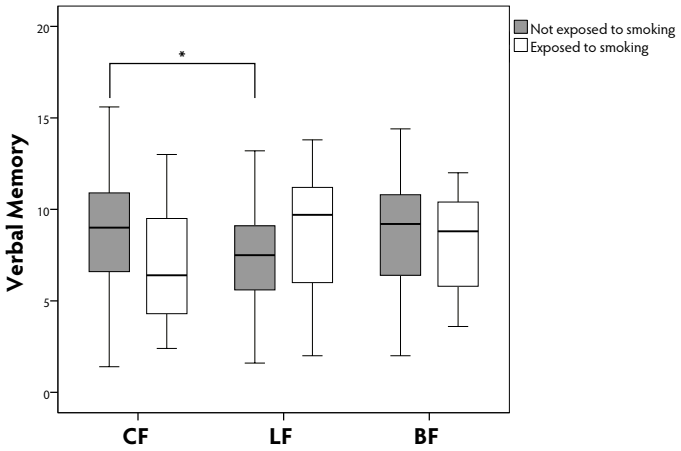

Figure 5. Verbal Memory: effect of early nutrition and prenatal exposure to maternal smoking Bold horizontal lines indicate median values (values provided); boxes represent interquartile ranges (IQRs); Vertical lines represent 1.5 IQR and small circles reflect outliers. * $p < 0.01$ when adjusted for confounders

3.4.5 Nutrition and language

Multivariable analyses indicated that scores on the language subscale of the NEPSY were not affected by infant nutrition (data not shown).

3.4.6 Nutrition and behaviour at home and at school

No evidence of a difference between the two formula groups nor between the formula groups and the BF group was found for the behavioural outcomes (data not shown).

3.5 Discussion

The present study indicates that in healthy term infants postnatal supplementation with LCPUFA for two months is not associated with a consistent beneficial effect on cognitive function at school age. Nevertheless, some effects were found, which were partially dependent on maternal smoking status during pregnancy. In children prenatally exposed to smoking LCPUFA supplementation was associated with positive effects on VIQ and learning and memory. In children whose mother had not smoked during pregnancy LCPUFA supplementation was associated with a negative effect on verbal memory. Also, LCPUFA supplementation was as-

sociated with a slightly lower mean performance on executive function. The domains affected by early nutritional modification involving LCPUFA, i.e., executive function and learning and memory and not language ability, were in line with our expectations. The direction in which outcome was affected by LCPUFA differed however from our expectations. The study confirmed the association between breastfeeding and better cognitive abilities for IQ.

A major limitation of the study is the 28% attrition. In longitudinal studies of this type a loss to follow-up is well-known and often inevitable,(25) attrition of less than 30% in 9 years may be viewed as relatively favourable. In addition two forms of selective attrition occurred: 1) a selective loss of children with optimal development in early infancy and 2) a selective loss of boys and children with a worse cognitive development at 18 months in the LF group. This means that from a developmental point of view the children studied may be considered as a slightly negative selection, and the LF children included in the follow-up a positive selection. The latter may imply that the minor negative effect of LCPUFA found in the present study might have been larger if our study had not suffered from selective drop-out of LF-children with a lower cognitive performance at 18 months. Attrition was not selective for smoking during pregnancy, the major modifier of the effect of LCPUFA supplementation on cognitive development.

Strengths of the study lie in the randomized design, the accurate prenatal and perinatal documentation, the long term follow-up period and the inclusion of an estimated maternal verbal IQ. Our study is the first to report effects of LCPUFA supplementation on cognition and behaviour at 9.

The effect of LCPUFA on cognitive function partially depended on the presence or absence of maternal smoking during pregnancy: supplemented children of non-smoking mothers had lower mean scores on verbal memory than control formula fed children, whereas supplemented children who were exposed to prenatal smoking had higher mean scores on VIQ and learning and memory than control formula children. Important to realize is that the negative effect of LCPUFA was relatively small (e.g. 3 points of IQ) whereas its positive effect in the infants exposed to prenatal smoking was about 6 points of IQ. The latter is close to half the standard deviation of 15. Such small differences may not be relevant for an individual but may be considered relevant on population level. The data confirmed that in infants receiving non-supplemented formula prenatal exposure to smoking is associated with a reduction of FIQ of approximately 10 points.(26) Previously Agostoni et al.(7) reported that smoking during pregnancy is associated with a diminished maternal capacity to synthesize AA and DHA. Our data suggest that a relative LCPUFA deficiency induced by maternal smoking during pregnancy may be compensated by the provision of LCPUFA after birth. This corresponds to the previously mentioned finding of Batstra et al.(5) who found a similar effect of breastfeeding.

Contrary to our expectations and to the assumptions in the literature(27) we also found minor negative effects of LCPUFA supplementation. A negative effect of LCPUFA supplementation was also reported by Van Goor et al,(28) who studied the effect of LCPU-

FA supplementation during pregnancy and lactation on neurological outcome in infancy. Additionally, the individual patient meta-analysis of Beyerlein et al(29) that did not find a statistically significant effect of postnatal LCPUFA supplementation on development, showed a trend towards a negative effect of LCPUFA on cognitive outcome. The data of these studies and the present data underscore that our understanding of fatty acid balances and their effect on the developing brain is limited.

Interestingly, the negative and positive effects associated with LCPUFA supplementation were found in the cognitive domains mediated by neural structures known to be sensitive to LCPUFA status.(12) The findings emphasize our limited understanding of the effect of LCPUFA on the developing human brain. Critical factors may be the timing of the nutritional intervention, and the balance between the various fatty acids that in the current make-up may not have optimally met the needs of the developing brain.(30,31)

Our study confirmed that breastfeeding is associated with improved cognitive abilities.(3) The data suggest that minor part of this association may be attributed to LCPUFA, as breastfeeding in children whose mother smoked during pregnancy was associated with a higher mean IQ in a similar way as LCPUFA-supplementation was associated with a higher mean IQ. In children not exposed to prenatal smoking (the majority of children), this association did not seem to play a major role. This fits with the notion that major part of the positive association between breastfeeding and cognitive abilities can be attributed to shared genome and maternal behaviour associated with breastfeeding practice.(3)

The present study warrants three conclusions. First, in healthy term infants no consistent beneficial effect of postnatal LCPUFA supplementation on cognitive function was found. Second, the data suggest a beneficial role of LCPUFA in the subgroup of children exposed to maternal smoking during pregnancy. Third, only minor part of the association between breastfeeding and higher IQ is mediated by LCPUFA.

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Declaration of author's roles: MHA initiated the follow-up study, CdJ and HK collected the data, CdJ, VF and MHA analyzed the data, CdJ and MHA drafted the report and HK and VF commented on drafts. All authors have seen and approved the final version.

Conflicts of interest: None

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Chapter 4

The Groningen LCPUFA study: no effect of short-term postnatal long-chain polyunsaturated fatty acids in health term infants on cardiovascular and anthropometric development at 9 years

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4.1 Abstract

Conflicting evidence exists on the effect of long-chain polyunsaturated fatty acid (LCPUFA) formula supplementation on cardiovascular health in term infants. It is known that LCPUFA supplementation does not affect infant growth, but long term outcome data are not available. The current study investigates whether two months LCPUFA formula supplementation affects cardiovascular and anthropometric development at 9 years. A prospective, double-blind, randomized control study was performed in healthy term infants: a standard formula control group (CF, n = 169) and a LCPUFA-supplemented group (LF, n = 145). A breastfed group (BF; n = 159) served as reference. At age 9, systolic and diastolic blood pressure, heart rate, head circumference, weight and height were measured. Univariate and multivariate analyses were performed. 63 to 79% of children were assessed. None of the cardiovascular or anthropometric measurements differed between the formula groups. Breastfed children had a marginally lower heart rate than formula fed children, in particular compared to children fed control formula. Blood pressure and parameters of growth including body mass index of breast and formula fed children did not differ. In conclusion, the study suggests that short term LCPUFA supplementation does not influence cardiovascular and anthropometric development at 9 years.

4.2 Abbreviations

AA:	Arachidonic Acid
BF:	Breastfed
CF:	Control Formula
DHA:	Docosahexaenoic Acid
LCPUFA:	Long-Chain Poly Unsaturated Fatty Acids
LF:	LCPUFA Supplemented Formula
OOS:	Obstetric Optimality Score

4.3 Introduction

The notion that breastfeeding has consistently been associated with a modest beneficial effect on blood pressure in later life is relevant for public health(1,2). The underlying mechanisms of the association are not clear. Long-Chain Polyunsaturated Fatty Acids (LCPUFA) may be candidates.

Until recently LCPUFA were not present in standard formula. Formula only contained the precursor essential fatty acids, alpha-linolenic acid (ALA,18:3n-3) and linoleic acid (LA, 18:2n-6). As young infants have a limited capacity to synthesize docosahexaenoic acid (DHA) and arachidonic acid (AA) from these precursors (3), LCPUFA levels in plasma or red blood cell membrane gradually decline in infants fed non supplemented formulae indicating a relative LCPUFA deficiency. Based on the advantageous effect of LCPUFA supplementation on neurodevelopment assessed at young age, it is advised that infant formulae should contain LCPUFA in quantities comparable to breastmilk(4). Consequently, most of the formulas currently on the market contain LCPUFA. However, there is still an ongoing debate concerning the long term consequences of dietary LCPUFA, including the effect on blood pressure regulation.

Studies on the effects of early LCPUFA supplementation on cardiovascular indices in humans, such as blood pressure and heart rate, are limited in number and heterogeneous in methodological quality (Table 1, 5–10). They also differ in the duration of supplementation, fatty acid dosage and lipid composition. Two studies on early postnatal supplementation with LCPUFA (5,6) and one in late infancy (7) reported beneficial effects of LCPUFA on blood pressure and heart rate in later life. Two other studies however found no effect of LCPUFA supplementation in early life (8,9) and one study even found a negative effect in school age boys(10).

Two recent meta-analyses indicated that early postnatal LCPUFA supplementation in term infants does not affect growth measures such as head circumference, weight, length and the resulting body mass index (BMI) until the age of 18 months(11,12). Breastfeeding on the other hand, is associated with a small but consistent reduction in obesity risk in later childhood (13). However, it is debated whether the effect is mediated by the nutritional contents of human milk or by associated factors, such as less maternal smoking during pregnancy and a maternal BMI < 25 (14).

The current study is part of the Groningen LCPUFA study, a randomized controlled trial on the effect of supplementation of formula with LCPUFA during the first two postnatal months in healthy term infants. The focus of the study is neurodevelopmental outcome, but also some other indicators of child health are evaluated. Previously the study reported that LCPUFA supplementation and breastfeeding were not associated with growth advantages at 18 months(12). Recently the participants of the study were re-assessed at 9 years. Here we report on cardiovascular health expressed as blood pressure and heart rate, and anthropometrics in terms of weight, length, BMI and head circumference.

Table 1.

Study set-up	Study Group	Supplementation with	FU age	Parameters used at evaluation	Effect of LCPUFA on cardiovascular health (95% CI)	References
FU of RCT	n=147 healthy FT	DHA 0.15-0.25 gr AA 0.3-0.4 gr per 100gr fat via formula birth till 4 mo	6 yr	Blood pressure	- lower mean blood pressure: 3 mm Hg (5.4 – 0.5) - lower diastolic blood pressure: 3.6 mm Hg (6.5 - 0.6)	(5)
Observational study	n=79 healthy FT	Contrasting: DHA 0.15% + AA 0.40% DHA 0.32% + AA 0.64%, via formula From 2 mo until 12 mo	6 mo	Resting heart rate, heart rate variability	- lower HR: presented in figure; numerical values not provided - lower values for heart rate variability: presented in figure; numerical values not provided	(6)
RCT	N=83 healthy FT	Fish oil 5mL daily From 9mo till 12mo	12 mo	Blood pressure, erythrocyte fatty acid composition and plasma lipid profile	- lower systolic blood pressure: 6.3 mm Hg (0.9 - 11.7) - increased erythrocyte (n-3) LCPUFA content - higher plasma total cholesterol: 0.51 mmol/L (0.07 - 0.95) - higher LDL cholesterol: 0.52 mmol/L (0.02 - 1.01)	(7)
RCT	N=98 healthy FT	Maternal fish oil: 1.5gr/day n-3, during the first 4 mo of lactation	7 yr	Blood pressure and body composition	Higher diastolic blood pressure: 6 mm Hg (CI not provided), boys only mean arterial blood pressure 6 mm Hg (CI not provided), boys only	(10)
RCT	N=73 healthy FT	Maternal fish oil: 4.5gr, during the first 4 mo of lactation	2.5 yr	Blood pressure and anthropometry	No effect	(9)
RCT	N=616	Diet change, additional n-3 fatty acids and reduced n-6 fatty acids in nutrition from time of weaning until 5 yr	8 yr	Blood pressure, arterial structure and function	No effect	(8)

FT = Full terms, FU = Follow-up, HR = Heart rate, mo = months, RCT = Randomized controlled trial, yr = year

The primary aim of the current paper is to explore whether the short term postnatal LCPUFA intervention was associated with blood pressure, heart rate and anthropometric development at 9 years. Additionally we explored whether breastfeeding was associated with a beneficial effect on blood pressure, heart rate and anthropometric development.

4.4 Methods

4.4.1 Subjects

The study is part of a double-blind randomized controlled trial investigating the effect of LCPUFA-supplementation on the development of healthy term infants (the Groningen LCPUFA study). Details on the study design, including exact diet composition, have been described previously (15). Between 1997 and 1999, expecting mothers were recruited during pregnancy checkup visits at the University and Martini Hospitals in Groningen and at midwife clinics in and near Groningen. Final enrollment occurred in the neonatal period. All infants were born at 37–42 wk of gestation. Mothers of 314 infants chose to bottle feed their child and 160 opted for breastfeeding. The infants receiving formula were randomized into a standard formula group (control formula, CF, n = 169) and a LCPUFA supplemented formula group (LF, n = 145). Standard formula consisted of Nutrilon Premium®. For the supplemented formula, the lipid fraction of Nutrilon Premium® was enriched with 0.45% (by wt) AA and 0.30% (by wt) DHA. The LCPUFA were provided as mix of phospholipids (15%) and triglycerides (85%) to mimic the composition of breastmilk. Supplementation lasted till the end of the second postnatal months. In case breastfeeding stopped prior to 2 months, the infant received LCPUFA-supplemented formula till the full age of 2 months. All formula-fed infants received control formula from two completed months until the age of 6 months. All 436 children (92% of the original groups) assessed at 18 months were eligible for re-examination at 9 years. At the 9 year follow-up, both parents and examiners were unaware of the type of formula-feeding the infant had received. The examiners were also blind to formula versus breast status.

4.4.2 Protocol and measurements

In the current follow-up the children and their parents were contacted and, depending on the wish of the participants, assessments were carried out either at the hospital or at home. Three hundred and forty one children agreed to participate: 91 in the LF-group (63%), 123 in the CF-group (73%) and 127 in the breastfed group (BF-group) (79%; Figure 1). During the study type of feeding was assessed prospectively by daily diaries filled in by the mothers until the infant was 8 months. Duration of breastfeeding varied from 1 to 56

weeks; 50% of mothers exclusively breastfed beyond 2 months. The assessments of cardiovascular parameters and anthropometrics were part of a more extensive evaluation focusing on neurodevelopmental outcome, see also(16).

Blood pressure and heart rate were measured immediately following a 15 minute rest period, measurement took place on the left arm with the child sitting on a chair and the arm resting on a table. An automated blood pressure monitor was used (Datascope Accutorr plus, Datascope corporation, Mahwah, USA) with a small adult cuff (10.6–23.9 cm size bladder; measuring with a precision of micrometers Hg). This procedure was performed twice during the assessment with an interval of an hour. In all children heart rate, systolic and diastolic blood pressure were recorded as the mean of the two readings.

Weight was measured using a calibrated radwag scale (Radwag, Radom, Poland; which measured with a precision of 500 milligrammes) and body length using a Seca stabilometer (Seca Deutschland, Hamburg, Germany; which measured with a precision of millimeters). BMI was determined using international standards, taking gender and age into account (17). Occipitofrontal head circumference was assessed using a nonstretchable 'lasso' tape which measured with a precision of millimeters.

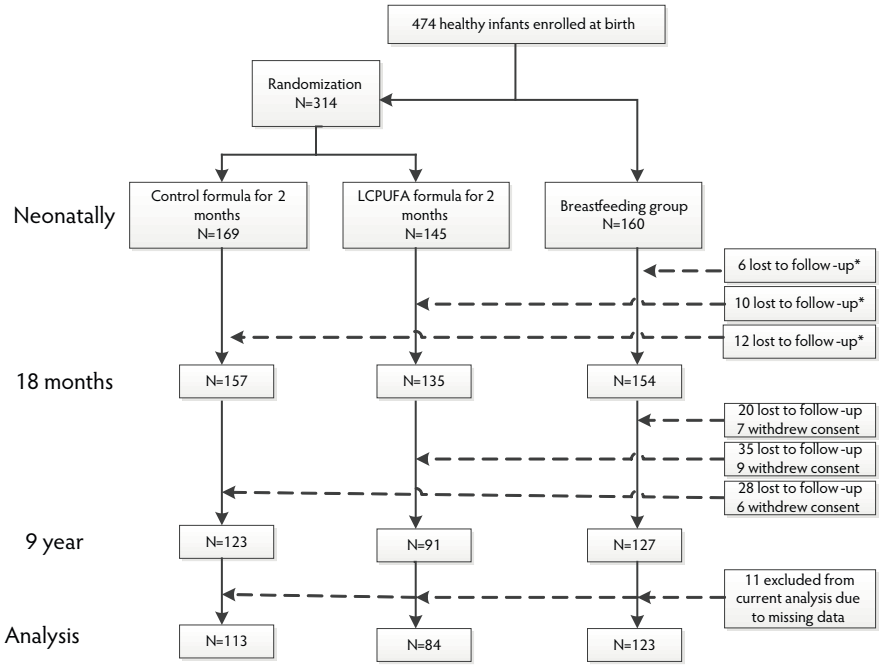

Figure 1. Flow diagram of children enrolled in the study and followed up until 9 y of age.
* for more detailed information see (21)

Data on pre- and perinatal conditions had been collected during enrollment with the help of the Obstetric Optimality Score (OOS).The OOS describes the obstetric conditions ranging from the parents' socio-economic status to the infant's condition immediately after birth (18). Anthropometrics had been recorded at birth and at 3 and 18 months (12). At the 9 year follow-up information was collected on parental education and profession, the child's medical history, nutritional habits and family composition.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Groningen University Hospital. Written informed consent was obtained from all subjects. The trial is registered under ISRCTN52788665.

4.4.3 Data analysis

The study was originally designed to detect a difference of 7.5 on the mental developmental index of the Bayley test of infant development at the age of 18 months between the LF and CF groups (assuming SD = 15, power 80% at 5% significance level). Accordingly, at least 57 children had to be included in each formula group.

Statistical analysis focused on differences between the two randomized groups, in addition differences between the formula groups and the breastfed group were analysed. The anthropometric and blood pressure data, apart from the children's BMI, were normally distributed. We used independent samples t-tests for crude comparison of the feeding groups and linear regression analysis for comparison adjusted for potential confounders. The children's BMI was dichotomized into normal (< = 25) / overweight (>25), chi-square tests were used for crude comparisons between the feeding groups and logistic regression analyses for comparisons adjusted for potential confounders.

Factors included into the multivariate analyses were variables associated with outcome at $p < 0.20$: gender, maternal level of education (1 = low, 2 = medium, 3 = high), smoking during pregnancy (yes or no), duration of the second stage of delivery, Apgar score at one and three minutes, birthweight, OOS and pre-pregnancy maternal body mass index (BMI). In line with other developmental studies, smoking was dichotomized into no/minimal smoking defined as <5 cigarettes per day and evident smoking defined as ≥ 5 cigarettes per day(19). Specific attention was paid to duration of exclusive breastfeeding as potential confounder. Anthropometric parameters at 3 and 18 months were not included in the multivariate analyses as they were not regarded as confounders but as outcome variables. A p-value of 0.05 or less was considered as statistically significant. Statistical analyses were performed using SPSS 14.0 for Windows (SPSS, INC, Chicago IL).

4.5 Results

In general, obstetric and social characteristics of children assessed at 9 years were comparable to those not participating in the follow-up at 9 years (Table 2 and Table 3). Nonetheless some selective attrition was present, the children who took part in the follow-up showed a less optimal neuromotor condition at 3 months ($p = 0.003$). Also, part of the attrition was group specific, more boys in the LF group did not partake (35 boys and 17 girls) compared to both the BF group (15 boys and 17 girls) and the CF group (24 boys and 17 girls). Additionally, in the LF group more children with a lower mental development index at 18 months were lost to attrition ($p = 0.007$; see (16)). Attrition was, however not selective for parameters of growth at birth and during infancy (Table 3).

Table 2. *Obstetrical and social characteristics of the three groups assessed at 9 years*

	CF group (n=123)	**LF group (n=91)**	**BF group (n=127)**
Gender, boys/girls	71 / 52	42 / 49	64 / 63
Maternal BMI, median (range)	24.7 (17.7 – 40.9)	23.7 (18.2 – 35.9)	22.6 (17.2 – 38.2)
Weight gain in kilograms during pregnancy, median (range) §	13 (0 – 46)	13 (0 – 26)	14 (0 – 31)
Presence of maternal smoking during pregnancy, n (%)§	28 (23%)	17 (19%)	13 (10%)
Presence of maternal hypertension during pregnancy, n (%)	21 (17%)	11 (12%)	11 (9%)
Birth weight (g), mean (SD)	3518 (473)	3527 (498)	3588 (436)
Apgar score after 3 minutes, median (range)	10 (7 – 10)	10 (7 – 10)	10 (5 – 10)
OOS, median (range)	59 (46 – 67)	59 (50 – 67)	60 (43-69)
Maternal education *			
- high[|], n (%)	15 (13%)	13 (15%)	60 (49%)
- medium[†], n (%)	85 (72%)	52 (60%)	57 (46%)
- low[‡], n (%)	18 (15%)	22 (25%)	6 (5%)

[|] University education or vocational college

[†] College graduate or junior vocational college

[‡] No education or primary education

* significant difference between all nutritional groups, found both in the groups lost to attrition and the groups assessed at 9: $p<0.01$

§significant difference between BF and FF in children assessed at 9: $p<0.05$

FF: Formula Fed

Table 3. *Growth, height and head circumference in the three nutritional groups a) weight (kg), b) height (cm), c) head circumference (cm).*

	CF		LF		BF	
	n	**Mean (SD)**	**n**	**Mean (SD)**	**n**	**Mean (SD)**
Weight at Birth	118	3.518 (0.473)	88	3.527 (0.50)	127	3.588 (0.436)
Weight 3 mo	95	6.306 (0.744)	80	6.323 (0.687)	120	6.253 (0.764)
Weight 18 mo	71	12.040 (1.40)	66	11.910 (1.40)	80	12.020 (1.430)
Weight 9 yr	116	34.0 (7.0)	85	33.70 (5.40)	124	33.10 (6.50)
Height at Birth	62	51.0 (2.2)	40	51.0 (2.2)	58	51.0 (2.0)
Height 3 mo	94	63.1 (2.6)	80	63.1 (2.6)	120	62.6 (2.6)
Height 18 mo	121	85.7 (3.5)	90	85.4 (3.7)	126	85.4 (3.8)
Height 9 yr	123	139.8 (5.3)	90	139.8 (5.9)	126	139.8 (6.2)
Head circumference Birth	57	35.0 (1.6)	38	34.6 (1.4)	43	34.9 (1.5)
Head circumference 3 mo	96	41.2 (1.5)	80	40.9 (1.3)	119	40.8 (1.3)
Head circumference 18 mo	119	48.4 (1.6)	90	48.1 (1.4)	125	48.4 (1.5)
Head circumference 9 yr	122	53.5 (1.7)	88	53.2 (1.6)	127	53.7 (1.6)

Systolic and diastolic blood pressure of LF-children did not differ from that of CF-children. Likewise, blood pressures of breastfed children did not differ from that of those who had received formula feeding (Table 4). Multivariate analysis confirmed these results (Table 5).

Table 4. *Blood pressure, heart rate and anthropometrics at 9 years*

	CF group		LF group		BF group	
	M (SD)	n	M (SD)	n	M (SD)	N
Systolic BP (mm Hg)	104.59 (7.9)	117	104.53 (9.0)	90	105.17 (7.8)	124
Diastolic BP (mm Hg)	63.78 (7.6)	117	62.27 (8.5)	90	63.77 (8.0)	124
Heart rate (beats/minute)	78.72 (10.3)	113	78.20 (9.2)	88	76.19 (9.6)	124
Head circumference (cm)	53.40 (1.6)	122	53.17 (1.6)	88	53.70 (1.6)	127
BMI (% normal)	72%	116	76%	85	79%	123

BP= Blood pressure

M= Mean

BMI was determined using international standards by Cole (17) taking age and gender into account

Heart rate of LF-children did not differ from that of CF-children (Table 4), but heart rate of the BF group was slightly lower than that of the CF group (p = 0.04; effectsize 0.914). Multivariate analyses confirmed the marginal difference (p = 0.049, 95%CI:-58.09 to -0.009; Table 5).

Weight, height, BMI and head circumference were similar in the three groups (Table 3). Multivariate analyses confirmed the lack of difference between groups.

Table 5. *Results of linear regression analysis of factors contributing to systolic blood pressure, diastolic blood pressure and heart rate*

	Heartrate		
Contributing factors:	**Effect**	**95% CI**	**P-value**
Type of feeding:			
LF vs CF	-0.540	-3.33 ; 2.24	0.70
BF vs CF	-2.550	-58.09 ; -0.009	0.049
BF vs LF	-2.0	-4.71 ; 0.70	0.146
Covariates:			
Neonatal Jaundice	7.54	-0.40 ; 15.48	0.063

Heart rate: reference CF: 78.57 n=325; R^2=0.24

CF group= Control group

LF group= LCPUFA supplemented group

BF group= Breastfed group

BMI= Body Mass Index

4.6 Discussion

Our data indicated that LCPUFA supplementation for the duration of two months in healthy term infants was not associated with a change in blood pressure, heart rate, weight, height, BMI or head circumference at 9 years. The study also revealed that children who were breastfed had a slightly lower heart rate at nine than children who had received formula.

A major limitation of the study is its attrition, which was 28%. However, considering the duration of the follow-up period, 9 years, this may be regarded as relatively favorable(20). Attrition was selective with respect to gender and cognitive development at 18 months in the LF group suggesting that LF-participants were a positive selection of the original LF group. However attrition was not selective with respect to social class and parameters of growth at birth and during infancy (Table 3). Another limitation of the study is its relatively weak power. Group sizes had been determined on the basis of neurodevelopmental outcome at 18 months measured with the Bayley Scales of Infant Development (21). Post-hoc power analyses indicated that with α set at 0.05 current sample sizes allowed for a detection of small effect sizes (0.20) with a power of 0.80, i.e., the groups allowed for the detection of differences in blood pressure of 1.5 mm Hg, in heart rate of 2 beats per minute, in body weight of 1.5 kg and in height of 1 cm. This means that the present study was not able to detect more subtle effects on growth, blood pressure and heart rate. It may also be regarded as a limitation that our parameters of cardiovascular health and anthropometrics were restricted to blood pressure, heart rate, bodyweight, length, BMI and head circumference and did not body composition, ECG and blood lipid profile.

The strengths of the study are its randomized design, the presence of information on a wide range of possibly confounding variables and its assessor-blinded evaluation.

Previous studies on the relationship between LCPUFA supplementation and blood pressure reported inconsistent findings: LCPUFA were associated with a positive effect on blood pressure, no effect or even a negative effect. This heterogeneity presumably is the result of the large variation in methodological quality of the studies, ranging from the use of high risk populations, high selective attrition, and co-intervention with other nutrients and secondly from variation between the studies in dosage and duration of the intervention and the ages during which the intervention was applied (see Table 1). The current study concludes that LCPUFA supplementation of formula for 2 months is not associated with blood pressure improvements of more than 1.5 mmHg and a decrease in heart rate of more than 2 beats per minute. This also implies that smaller effect sizes nor larger effects of longer periods of supplementation are not precluded. Originally our study was supposed to be one of a series in which also the effect of longer periods of supplementation were to be evaluated. However, our short term supplementation study was the only one that was completed.

Breastfeeding on the other hand, has shown a relatively consistent relation with blood pressure: it is associated with a modest decrease in blood pressure, i.e. a reduction of around 1.4 mm Hg in systolic blood pressure and 0.4 mm Hg in diastolic blood pressure

(1). These breastfeeding effects are smaller than the current study was able to detect. However we did find suggestions of a minor advantage for breastfeeding in heart rate, the other parameter of cardiovascular health. The minor reduction in heart rate may reflect a slight shift in autonomic control involving a minor decrease of sympathic dominance. This may be associated with a minor reduction in risk for cardiovascular diseases in adulthood (22). The small size of the effect is illustrated by the fact that the advantage only reached statistical significance in the comparison with control formula. Our finding of a minor beneficial effect of breastfeeding on cardiovascular indices is in line with other reports but somewhat smaller than often reported. This might be explained by the relatively short average period of breastfeeding, 53% of breastfed infants received a maximum of 8 weeks exclusive breastfeeding (23), which is representative of Dutch breastfeeding habits (24).

None of the anthropometric measures in this study were associated with early postnatal nutrition. The lack of effect of postnatal LCPUFA supplementation is in line with existing literature, but the lack of difference between the breastfed and the formula fed groups is not. This might be explained by the limited ability to detect differences between the groups and to the above mentioned breastfeeding practices in the Netherlands.

To summarize , the current study suggests that LCPUFA supplementation of formula in healthy term infants for the duration of two months does not influence cardiovascular and anthropometric development at the age of 9 years. Breastfeeding is found to have a marginal beneficial effect on cardiovascular development. The study underscores the need for carefully designed and well controlled studies on the effect of LCPUFA supplementation on cardiovascular development.

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Chapter 5

Neonatal fatty acid status and neurodevelopmental outcome at 9 years

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Submitted

5.1 Abstract

Long-Chain Poly Unsaturated Fatty Acids (LCPUFA) are important for prenatal brain development, previous studies assessed outcome until 7 years. In the current study the relationship between fatty acid status at birth and neurodevelopment at 9 is investigated.

An observational (cohort) study was performed as a secondary analysis of data from a prior randomized trial on effects of postnatal LCPUFA including 235 children. Docosahexaenoic acid (DHA), arachidonic acid (AA) and trans fatty acid concentrations in umbilical vessel wall were determined. Neurological condition, cognition and behaviour were assessed at 9. Multivariate analyses were carried out to adjust for potential confounders. 74% of the original study group was assessed at 9 years. Children with complex minor neurological dysfunction showed lower DHA values than children with better neurological condition. Two specific types of neurological dysfunction were associated with lower venous DHA values: dysfunctional posture and tone regulation and dyskinesia. Neonatal AA values were not associated with neurological outcome. Neither neonatal DHA nor AA values were associated with cognition and behaviour at 9. Venous and arterial trans fatty acid values were positively associated with selective attention. We conclude that DHA status at birth showed a significant positive association with neurological condition at 9 years. AA status at birth was not associated with neurodevelopment at 9 years.

5.2 Introduction

The human nervous system increases rapidly in size and complexity during pregnancy. Adequate supply of necessary nutrients such as Long-Chain Poly Unsaturated Fatty Acids (LCPUFA) to the fetus is essential for optimal neurodevelopment.

Studies on the effects of docosahexaenoic acid (DHA) or fish oil supplementation during pregnancy on neurodevelopmental outcome provided inconsistent results (1-6). Observational studies on the relationship of neonatal DHA, arachidonic Acid (AA) and essential fatty acid (EFA) status with neurological outcome from preschool age to 7 years reported associations with neonatal DHA status only (7-9). Most studies using cognitive development as outcome measure demonstrated no associations with neonatal LCPUFA status (7,10,11), except for the study of Boucher et al (12). The latter study reported a significant positive association between neonatal DHA and memory at school age. Data on the relation between behavioural development and LCPUFA are scarce. The only study that we are aware of (13) reported no associations between neonatal LCPUFA status and behaviour at preschool age.

Little is known on the effects of trans fatty acids (TFA) on human development; however, the negative association between TFA and LCPUFA suggests a potentially adverse effect (14). This hypothesis was supported by two studies that reported negative associations between TFA and neurological condition in infancy and preschool age (7,15).

The current study is part of the Groningen LCPUFA study, a randomized controlled trial on the effect of postnatal supplementation of LCPUFA on neurodevelopment. The study revealed that postnatal LCPUFA supplementation was not associated with a consistent beneficial effect on neurodevelopmental outcome at 9 years (16,17). In a subgroup of 317 infants of the LCPUFA-cohort also fatty acid status in the umbilical cord was analyzed. The aim of the present paper is to evaluate in those healthy term infants the relationship between the relative DHA, AA and TFA contents in umbilical vessel wall lipids and neurological, cognitive and behavioural outcome at 9 years. Primary outcome of the current study is neurological condition in terms of minor neurological dysfunction (MND). Secondary outcome parameters were the so-called neurological optimality score (NOS), various measures of cognition and behaviour. Specific attention was paid to possible interactions between neonatal fatty acid status and a) sex, as a previous study suggested that in particular girls may benefit from early DHA supply (18) and b) smoking during pregnancy, which is known to interact with postnatal LCPUFA supplementation (16).

5.3 Subjects and Methods

5.3.1 Study population and design

The study is an observational (cohort) study, performed as a secondary analysis of data from a double-blind randomized controlled trial investigating the effect of LCPUFA-supplementation on development in healthy, term infants (the Groningen LCPUFA study; for details see Bouwstra et al (19)). Pregnant women were recruited between February 1997 and October 1999; from a total of 474 mother infant pairs, 314 infants were bottle fed and 160 breastfed. The infants receiving formula were randomized into a standard formula group (control formula, CF, n = 169) and a LCPUFA supplemented formula group (LF, n = 145). The standard formula was Nutrilon Premium (Milupa, Friedrichsdorf, Germany). For the supplemented formula, the lipid fraction of Nutrilon Premium® was enriched with 0.45% (by wt) AA and 0.30% (by wt) DHA. The duration of supplementation was 2 months. In case breastfeeding stopped prior to 2 months, the infant received LCPUFA-supplemented formula till the age of 2 months. All formula-fed (FF) infants received control formula between 2 and 6 months.

Parents of 317 infants gave permission to obtain umbilical cord tissue (67% of the original population, see Figure 1).

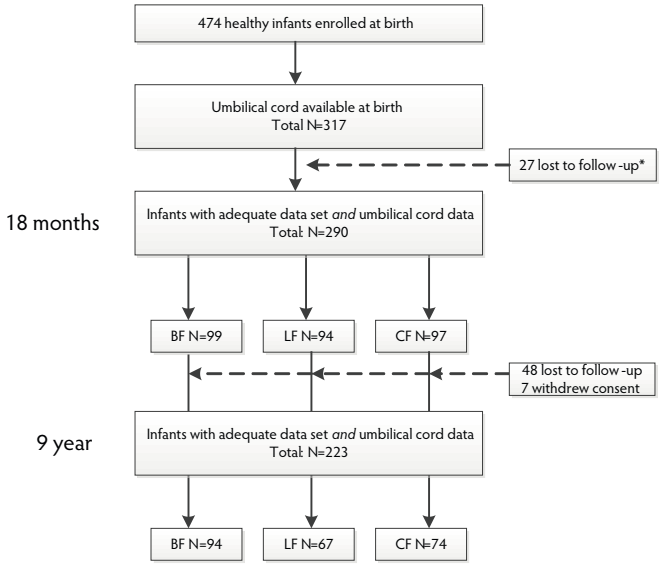

Figure 1. Flow diagram of children enrolled in the study and followed up until 9 years of age.
* For more detailed information see[7]

The umbilical cord was collected shortly after parturition. Seven to 10-cm samples were taken at the most proximal site of the placenta and stored in saline at 4°C for a maximum duration of 24 hour until further processing. Details on collection and processing can be found in Muskiet et al (20). Neurological condition was assessed at 3 and 18 months. All 290 children assessed at 18 months (91.5% of the original groups) were eligible for re-examination at 9 years. At the 9 year follow-up, both parents and examiners were unaware of the type of formula-feeding the infant had received and of neonatal fatty acid status. The examiners were also blind to formula versus breast status. Depending on the wish of the participants, the assessment was carried out in the hospital or at home.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Ethics Committee of the Groningen University Hospital. Written informed consent was obtained from all subjects. The trial is registered under ISRCTN52788665.

5.3.2 Analysis of the fatty acids of the umbilical vessels

Data were expressed as percentages of weight of fatty acids with a chain length of 14-24 carbon atoms. DHA and AA status were based on their respective levels. TFA status was derived from the sum of t16-1n7 + tt18-2n6 + tc18-2n6+ ct18-2n6 + t18-1n9/7 (21).

5.3.3 Neurological assessment

Neurological condition of the children was evaluated with the examination of Touwen (22), which is a standardized, age specific assessment designed for the assessment of minor neurological dysfunction (MND (23)). Essential in the diagnostics of MND is the presence of coherent clusters of signs, single signs do not have clinical significance, signs only have significance when they co-occur (cluster) with other signs within a functional domain. The examination is organized into eight functional domains: posture and muscle tone, reflexes, dyskinesia, coordination, fine manipulative ability, associated movements, sensory deficits and cranial nerve functioning. The examination results in a clinical classification and in a Neurological Optimality Score (NOS). The clinical classification consists of four categories: normal, simple MND, complex MND, or abnormal. A child is considered neurologically abnormal in the presence of a clear neurological disorder such as cerebral palsy. Simple MND denotes the presence of one or two domains of dysfunction and is present in about 15-20% of children. It has little clinical relevance and can be regarded as a sign of typical but non-optimal brain functioning, in other words as a minor neurological difference. Complex MND denotes the presence of more than two domains of dysfunction and is the clinically relevant form of MND. In an etiological

sense complex MND can be considered a borderline form of cerebral palsy as it is linked to pre- and perinatal adverse events (24). A child is classified as neurologically normal when no domains are scored as deviant or in case of the isolated presence of a mild dysfunction in reflex activity.

The NOS is a quantitative expression of neurological condition. It uses the optimality concept to provide a sensitive measure of the child's overall neurological status. For 64 items, representing the entire neurological examination, an optimal range was identified (see (17,23)). The total number of items with a value within the predefined optimal range forms the neurological optimality score of a child. Note that there is a conceptual difference between normality and optimality, as the range for optimal behaviour is narrower than that of normal behaviour (25).

The neurological examination according to Touwen has a good intrarater, interrater and test-retest reliability, the kappa statistics of the three forms of reliability for neurological classification ranges between 0.71 and 0.83 (26). Its construct validity is reflected by the differential relationship for simple and complex MND with prenatal and perinatal adverse events: adverse conditions during early life have a weak to moderate relationship with simple MND and a strong correlation with complex MND (24). Also predictive validity is good; for instance the severity of MND at 9 years predicts the chance of learning and behavioural problems at 14 years (24,27).

5.3.4 Assessment of cognition

Assessment of cognition consisted of a battery of tests, including intelligence testing and the evaluation of specific cognitive functions. Intelligence was assessed using the WASI – Wechsler Abbreviated Scale of Intelligence (28), resulting in a Full IQ (FIQ) score. Specific cognitive performance was assessed by means of the NEPSY, the Tea-Ch and the Children's Memory Scale. The NEPSY (29) is designed to reliably assess neuropsychological functioning in 3 to 12 year-old children in a maximum of five domains. To prevent possible overlap with other parts of the assessment battery, testing was limited to a representative selection of tests in the following three domains: 1. Attention and Executive Functions, 2. Memory and Learning Ability and 3. Language Ability. The Tea-Ch – Test of Everyday Attention for Children is a standardized and valid test for the assessment of attention in children aged 6 to16 years (30). All three domains were assessed: 1. Selective Attention 2. Sustained Attention and 3. Attentional Control/Switching. In addition, verbal memory was assessed by the Word Pair subtask of the Children's Memory Scale. For WASI, NEPSY, Tea-Ch and the Children's Memory Scale the original norms were used as no Dutch norms were available.

5.3.5 Assessment of behaviour

Behaviour was evaluated by means of parental and teacher's questionnaires, i.e., the Dutch versions of the Children's Behavioural Check List (CBCL; (31)) and the Teacher Report Form (TRF; (31)).The reliability and validity of CBCL and TRF are well established (31,32). Outcome parameters were the composite scales for internalizing, externalizing and total problems.

5.3.6 Assessment of potential confounders

Data on pre- and perinatal conditions had been collected during enrolment with the help of the Obstetrical Optimality Score (OOS).The OOS describes the obstetrical conditions ranging from the parents' socio-economic status to the infant's condition immediately after birth (33). At the assessment of 18 months, maternal verbal intelligence (IQ) was estimated using a very abbreviated version of the Wechsler Adult Intelligence Scale (WAIS III), limited to the subtest on information and vocabulary (34). At the 9 year follow-up, information was collected on parental education and profession, the child's medical history, family composition and nutritional habits.

5.3.7 Statistical analysis

The cognitive parameters were normally distributed, but none of the other variables were normally distributed. The NOS was normalized by transformation to the fifth power. To evaluate the differences in fatty acid composition of the umbilical vein and artery between children with complex MND and those with a better neurological condition (normal or simple MND) and between those with or without dysfunction in a specific neurological domain, univariate and multivariate logistic regression analyses were performed. The relations between prenatal fatty acids and NOS and the relations between prenatal fatty acids and cognitive tests were analyzed by use of Spearman correlations and linear regression analyses. For the univariate analysis of relationships between neonatal fatty acid status and the behavioural data Spearman's rho was applied. For the multivariate analyses the behavioural data were dichotomized into normal versus borderline range according to Dutch norms (31), and analyzed by means of logistic regression analyses. An important potential mediator taken into account in the multivariate statistics was postnatal nutritional group (LCPUFA supplemented formula, control formula or breastfeeding). In addition, we considered the following variables as potential confounders: maternal estimated verbal IQ, sex, maternal level of education (low versus medium/high), smoking during pregnancy (yes or

no), pre-pregnancy maternal body mass index (BMI) and obstetric optimality score (OOS). In line with other developmental studies, smoking was dichotomized into no/minimal smoking defined as <5 cigarettes per day and evident smoking defined as ≥ 5 cigarettes per day (35). Specific attention was paid to the presence of sex – fatty acid status and smoking during pregnancy – fatty acid status interactions (see (16)), if an interaction with sex is found the data will be presented for each of the sexes separately. All variables were entered into the models. A p-value of 0.05 or less was considered as statistically significant. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, INC, Chicago IL).

5.4 Results

Two hundred and thirty five of the 290 children who were assessed at 18 months and of whom data on neonatal fatty acid status were available agreed to participate in the assessment at 9 years (74.1% of the original study group consisting of 317 children, Figure 1). Obstetrical data of the study groups and sociodemographic characteristics of the parents are described in Table 1. In general, neonatal fatty acid status, obstetrical and social characteristics of the children who were and who were not assessed at 9 years were comparable. Yet, children who received LCPUFA supplemented formula were less likely to participate in the 9-year-follow-up than the children who received other postnatal nutrition ($p < 0.01$) and maternal verbal IQ of children that participated at 9 yr was significantly higher than those that did not participate ($p = 0.025$). Also, children who did not take part in the follow-up at 9 year showed lower scores on mental and psychomotor indices of the Bayley Scales of Infant Development than those who did participate (mental index: $p = 0.004$, median difference 3.5 points; psychomotor index: $p = 0.005$, median difference 3 points). None of the neurological, intelligence or behavioural outcomes at 9 years were affected by the site of investigation (in the hospital or at home), or by duration of exclusive breastfeeding. No interactions between smoking during pregnancy and fatty acid status were found (data not shown). Statistical description of the variables can be found in Table 2.

5.4.1 DHA and AA

Univariate analysis demonstrated that DHA in both the umbilical vein and artery were significantly lower in the group of children with complex MND than in the groups of neurologically normal children and children with simple MND (vein $p = 0.007$, OR: 0.413; artery $p = 0.044$, OR:0.591 Figure 2). Both relations remained statistically significant after correction for confounders; however, the association between complex MND and venous DHA was restricted to the boys (Table 3). Two specific neurological domains showed an association with neonatal fatty acid status. The children – both boys and girls – with dysfunctional posture and muscle tone regulation and the children with dyskinesia had

a lower concentration of DHA in the umbilical vein than the children without these specific dysfunctions (posture and tone p = 0.023, OR = 0.55; dyskinesia p = 0.017, OR = 0.20). The relations remained statistically significant after correction for confounders (Table 3). The association between neonatal DHA status and neurological condition was also reflected in the NOS. Multivariate analysis confirmed the association between venous DHA and NOS (Table 3). Please note that the NOS used in Table 3 has the transformed scale. An effect size of 0.16, as is reported in Table 3, can therefore be interpreted on the original scale as follows: a NOS value of 57 transforms to (57/50)5 = 1.9254. Increasing this by 0.16 gives 2.0854. Transforming this back to the original scale results in 50 x 2.08540.2 = 58.0. This means that a change of 1 point in venous DHA is associated with a change of 1 point on the original NOS scale. The association was similar for boys and girls. Neonatal DHA status was not associated with cognitive and behavioural outcome. Neonatal AA was not associated with neurological condition, cognitive and behavioural outcome Interestingly, postnatal nutritional group did not play a significant role in any of the observed associations between neonatal LCPUFA status and neurodevelopmental outcome at nine.

Table 1. *Obstetrical and social characteristics of the infants assessed at 9 years.*

Variable	Study group	Did not participate
No. of participants with umbilical cord data assessed at 18 months (%)	235 (74.1%)	82 (25.9%)
Nutritional group**		
- control formula (CF), n (%)	74 (31.5%)	30 (36.6%)
- LCPUFA supplemented formula (LF), n (%)	67 (28.5%)	35 (42.7%)
- breastfed group (BF), n (%)	94 (40.0%)	17 (20.7%)
Gender, n (%) boys	125 (53.2%)	48 (58.5%)
Birth weight (g), mean (SD)	3547.5 (438.4)	3545.48 (400.3)
Maternal education		
- high (university education or vocational college), n (%)	48 (20.4%)	12 (14.6%)
- medium (college graduate or junior vocational college), n (%)	126 (53.6%)	36 (43.9%)
- low (no education or primary education), n (%)	42 (17.9%)	18 (21.9%)
Presence of maternal smoking >5cig/day during pregnancy, n (%)	38 (16.2%)	11 (13.4%)
Maternal BMI before pregnancy >18.8 & <24.2, n (%)	128 (54.5%)	43 (52.4%)
Maternal VIQ*, mean (SD)	116.8 (14.9)	112.0 (16.4)
OOS, median (range)	59 (21)	59 (22)

* significant difference p = 0.025

** significant difference p < 0.01

OOS = Obstetrical Optimality Score

Table 2. *Description of variables used in the statistical analyses.*

Independent variables:	
DHA vein, median (25th , 75th percentile)	4.24 (3.78 , 4.77)
DHA artery, median (25th , 75th percentile)	4.42 (3.99 , 5.05)
AA vein, median (25th , 75th percentile)	16.53 (15.63 , 17.27)
AA artery, median (25th , 75th percentile)	12.76 (11.69 , 13.97)
TFA vein, median (25th , 75th percentile)	0.703 (0.57 , 0.80)
TFA artery, median (25th , 75th percentile)	0.67 (0.57 , 0.78)
Dependent variables:	
MND, % normal, % simple MND, % complex MND	49.4 , 37.9 , 12.8
NOS, median (25th , 75th percentile)	57 (55 , 59)
FIQ, mean (SD)	101.25 (12.75)
Attention and Executive Functions, mean (SD)	10.26 (2.50)
Memory and Learning, mean (SD)	8.82 (2.24)
Language Ability, mean (SD)	9.86 (2.22)
Selective Attention, mean (SD)	7.53 (2.68)
Sustained Attention, mean (SD)	7.91 (2.95)
Attentional Contol/Switching Attention, mean (SD)	8.41 (2.32)
Verbal Memory, mean (SD)	8.33 (3.10)
CBCL Internalizing problems, median (25th , 75th percentile)	52 (46 , 59)
CBCL Externalizing problems, median (25th , 75th percentile)	51 (41 , 57)
CBCL Total problems, median (25th , 75th percentile)	51 (45 , 58)
TRF Internalizing problems, median (25th , 75th percentile)	52 (45.5 , 55)
TRF Externalizing problems, median (25th , 75th percentile)	51 (43 , 57)
TRF Total problems, median (25th , 75th percentile)	50.5 (47 , 55)

Table 3. *Results of logistic regression analysis of the contribution of DHA to the complex form of Minor neurological Dysfunction (MND), domain of dysfunctional Posture and Tone and domain of Dyskinesia and of the linear regression analyses of the contribution of DHA to the Neurological Optimality Score (NOS).*

Contributing factors:	Complex MND OR (95%CI)	Dyskinesia OR (95%CI)	Posture and Tone OR (95%CI)	NOS Effect (95%CI)
Venous DHA in total group		.119 (.021 , .675)*	.425 (.223 , .811)**	.16 (.055, .264)**
Venous DHA within boys	.220 (.079 , .614)**			
Venous DHA within girls	.536 (.176 , -1.63)			
Arterial DHA in total group	.392 (.206 , .745)**	.344 (.118 , 1.006)	.596 (.353 , 1.007)	0.109 (.010 , .208)*

The data were adjusted for the following confounders: postnatal nutritional group, gender, OOS, maternal verbal IQ, maternal education, maternal smoking during pregnancy, maternal BMI before pregnancy

* significant difference $p < 0.05$

** significant difference $p < 0.01$

5.4.2 Trans fatty acids

Neonatal trans fatty acid status was not associated with clinical neurological classification or specific neurological domains of dysfunctions. However, venous and arterial trans fatty acid status was positively associated with selective attention. Multivariate analyses confirmed these associations (Table 4). Trans fatty acid status was not associated with other cognitive functions, including other types of attention, and with behaviour at 9 years.

Table 4. *Results of linear regression analyses of the contribution of neonatal venous and arterial trans fatty acid status to selective attention.*

Contributing factors	Effect (95% CI)
Venous trans fatty acids	2.18 (.601 , 3.755)**
Arterial trans fatty acids	2.71 (1.24 , 4.19)**

The data were adjusted for the following confounders: postnatal nutritional group, gender, OOS, maternal verbal IQ, maternal education, maternal smoking during pregnancy, maternal BMI before pregnancy.

** significant difference $p < 0.01$

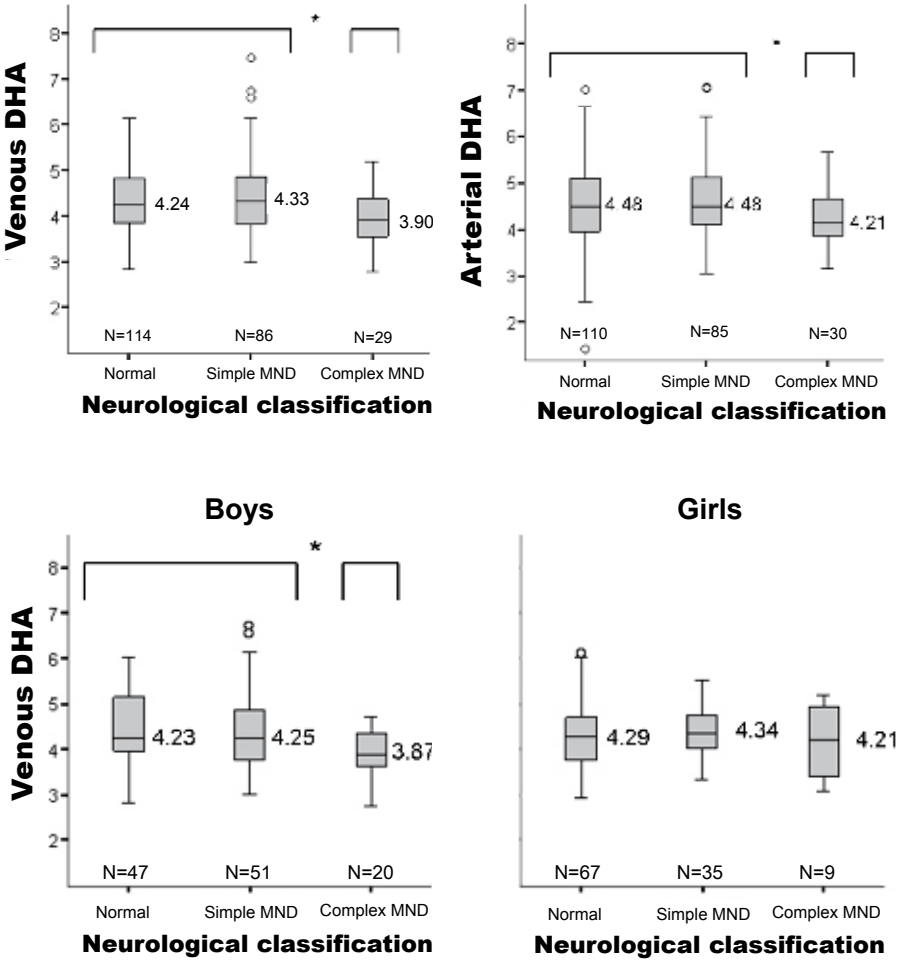

Figure 2. Neonatal arterial and venous DHA in the whole group (upper panels) and neonatal venous DHA separately for boys and girls. The horizontal lines represent median values, the boxes the interquartile ranges and the vertical lines the range of values except outliers which are depicted by small dots.

* denotes statistically significant difference; differences between the neurologically normal and simple MND vs complex MND: whole group venous DHA p = 0.007, whole group arterial DHA p = 0.024, restricted to boys venous DHA p = 0.010.

5.5 Discussion

The present study indicated that the previously demonstrated association between prenatal DHA and neurological development extends at least till the age of 9 years. Higher levels of venous DHA were related to less choreiform dyskinesia, less problems in posture and muscle tone regulation, a more optimal NOS, and less complex MND in boys. Arterial DHA was also associated with less complex MND, but in both sexes, and in a more optimal NOS in both sexes. Higher levels of TFA in both umbilical cord vessels were related to better selective attention scores.

The positive association between DHA and neurological development in the present report is in agreement with literature (8,9) and is in line with previous results from our study (7). The association suggests that presence of higher neonatal levels of DHA protects against clinically relevant complex MND. Detailed analysis revealed that in particular the neurological domains choreiform dyskinesia and posture and muscle tone were associated with neonatal DHA status. These domains are mediated predominantly by subcortical circuits in which the striatum plays a major role. Interestingly, animal research demonstrated that these circuits accrue the largest amounts of DHA during early development (36).

The protective effect of DHA on the development of minor neurological dysfunction showed a minor sex effect: a higher venous DHA status was related to less complex MND in boys but not in girls. Sex differences in neurological development have been reported before (18,37). The current difference may be attributed to the well-known increased vulnerability of boys for neurodevelopmental disorders and MND (37).

Interestingly, neonatal DHA status was not related to intelligence or any other measure of cognition. Intelligence is attributed especially to activity in frontoparietal networks (38), parts of the cortex which accrue relatively little DHA during early development (36). In line with literature, no associations between umbilical AA levels and development at school age are found. This suggests a temporary effect of prenatal AA only.

TFA have been associated consistently with less optimal health in the adult population (39). In infants and children, higher TFA levels have been related to reduced birth weight and reduced child growth (14,40). As mentioned in the introduction, limited studies have been performed relating TFA to neurological development, yet they point to a negative relation with neurological development. In the present study a minor positive association has been found with selective attention. We are unable to offer a biologically plausible explanation for this association and suggest this to be a chance finding.

The present study demonstrated an association between neonatal LCPUFA status and neurological development at 9, while taking into account postnatal LCPUFA supplementation as a possible mediator. As the postnatal LCPUFA supplementation did not contribute significantly to neurological outcome (17) this implies that especially prenatal LCPUFA status affects neurological development. Prenatal LCPUFA status depends on maternal LCPUFA consumption during pregnancy and maternal pre-pregnancy LCPUFA

status (41). The lack of evidence for a consistent beneficial effect of LCPUFA supplementation during pregnancy on neurological development(1) suggests that maybe the woman's pre-pregnancy LCPUFA status matters more for the development of her child's brain than LCPUFA consumption during pregnancy. Another intriguing observation is that we did not find an interaction between smoking during pregnancy and neonatal fatty acid status. This contrasts with the previously reported interaction between smoking during pregnancy and postnatal LCUPFA supplementation on cognitive development: children prenatally exposed to LCPUFA supplementation benefited in some cognitive domains from postnatal LCPUFA supplementation, whereas children not exposed to maternal smoking did not (16). It is conceivable that the direct competing effect of prenatal smoking abolishes the potentially additional positive effect of LCPUFA on neurodevelopment.

The present study is a secondary analysis of data from a randomized controlled trial on formula supplementation. This may be considered a limitation as the supplementation could have acted as a mediator. Yet, the multivariate analyses showed that postnatal nutritional group did not affect neurodevelopmental outcome. A secondary limitation is the sample size, this may be considered too small to draw strong conclusions. A more serious limitation of the present study is the attrition of 25.9%. However, a certain amount of attrition is considered inevitable in longitudinal studies (42) and attrition of less than 30% over a time period of 9 years may be considered acceptable. Unfortunately, attrition was selective, as children taking part in the present follow-up had higher scores on the developmental tests at 18 months.

The strengths of the study are the long term follow-up period, the detailed prenatal and perinatal documentation, the elaborate neurological, cognitive and behavioural test battery and the multivariate analyses with a large set of possible confounders, including smoking during pregnancy and estimated maternal verbal IQ.

In conclusion, the present study showed that higher neonatal DHA levels are associated with a more optimal neurological development at 9 years of age. Neonatal DHA was not associated with cognition or behaviour at 9 and neonatal AA was not associated with any neurodevelopmental parameter at 9. The study suggests that prenatal DHA levels have a larger impact on neurological development than postnatal DHA levels.

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Chapter 6

General Discussion

It is clear that LCPUFA are important elements of the human nervous system. However their exact role in human development, and more specifically the effect of early postnatal AA and DHA supplementation on the development of the nervous system at age 9 as well as the relation between neonatal DHA, AA and trans fatty acid concentrations and development of the nervous system at age 9 had not been assessed.

In the current follow-up no consistent effects of postnatal AA and DHA supplementation on neuro-motor, cognitive, behavioural, anthropometric and cardiovascular development were found. Looking at the separate indices, we found that none of the neuromotor indices was related to AA and DHA supplementation. The effects on cognitive tests were complex due to an interaction with maternal smoking during pregnancy. In children exposed to smoking during pregnancy LF was associated with higher scores on verbal IQ and learning and memory, in children not exposed to smoking during pregnancy LF was associated with lower scores on verbal memory. The whole LF group scored worse than the CF group on executive function. With respect to the cardiovascular and anthropometric indices, no relations with postnatal LCPUFA supplementation were found.

Breastfeeding was related to better fine manipulative abilities. It was also to some extent associated with a higher IQ, breastfed children demonstrated a higher IQ than children who had received control formula and had been exposed prenatally to smoking. In addition, breastfed children showed a marginally lower heart rate than formula fed children.

Neonatal DHA levels were associated with neuro-motor development: children with complex minor neurological dysfunction showed lower mean DHA values than children with better neurological condition, for the venous DHA levels this effect was restricted to the boys. The neurological domains which were associated in particular with lower venous mean DHA values were dysfunctional posture and tone regulation and dyskinesia. Neonatal AA values were not associated with neurological outcome. Neither neonatal DHA nor AA values were associated with cognition and behavior at age 9. Trans fatty acid levels were positively associated with selective attention, both in the vein and in the artery.

6.1 Effects of postnatal LCPUFA supplementation

The relation between neurological structure development and function is far from clear cut. This means that it is quite difficult to relate the effects of LCPUFA supplementation on a neuronal level to enhanced or possibly diminished test results in children. The baboon study by Diau (1) demonstrated that LCPUFA accumulate mainly in the cortical grey matter, predominantly in the synaptic membranes, and to a lesser extent in the white matter. The study by Diau also demonstrated that the highest concentrations of LCPUFA were found in the basal ganglia, precentral, postcentral, prefrontal and occipital cortices, hippocampus, thalamus and cerebellum. This indicates that LCPUFA intake might affect in particular circuitries involved in sensorimotor integration, motor learning, intelligence,

executive functions, attention, and memory (2). Other animal studies indicated that LCPUFA status in early life may affect dopaminergic and serotonergic circuitries. Functions subserved by these systems are for instance attention regulation, aggression and mood (3).

In the current study effects of postnatal LPUFA supplementation were found on verbal IQ, learning and memory, verbal memory and executive function (although the latter was a negative effect). No associations have been found with neuro-motor development, language function, full IQ, performance IQ, selective attention, sustained attention, switch attention, cardiovascular and anthropometric indices.

The cognitive functions that were associated with LCPUFA supplementation are in line with the work of Diau. However, based on that work more functions could have been expected to be associated with the LCPUFA association. Also, not all findings are in the expected direction. The cognitive and neuro-motor functions tested in the current study are complex tasks. Complex tasks are the result of the performance of networks of brain regions that integrate vast amounts of information. The developmental process of such networks requires the coordination and integration of a multitude of factors, among which genetic programs, environmental exposures and personal experiences. Nutrition is an important factor, however it is but one of the many factors that influence neuronal development. This could imply that the influence of nutrition may be diluted. Also, the study by Diau demonstrated that not all regions are equally influenced by LCPUFA supplementation (1). This suggests that even though important parts of a network may profit from higher LCPUFA levels, the entire network may not show a related improved functioning due to limitations in other parts of this network that have not profited from this advantage. Last but not least the developing baboon brain is not identical to the developing human brain.

The effects of LCPUFA supplementation depended for a large part on maternal smoking. Children having been exposed to maternal smoking during pregnancy (meaning more than 5 cigarettes per day) showed a positive effect of LCPUFA supplementation on VIQ and learning and memory. Children that were not exposed to maternal smoking during pregnancy showed negative effects of LCPUFA supplementation on verbal memory. According to work by Agostoni (4) maternal smoking during pregnancy causes diminished LCPUFA levels in infants, both omega 6 and omega 3, as was measured shortly after birth. This may be related to the IQ drop of 8 points that was found as a result of smoking during pregnancy in the current study (see Table 2 chapter 3). Supplementing LCPUFA via formula may partly compensate for this detrimental effect, something that has been suggested in literature (5).

No associations were found between LCPUFA supplementation and blood pressure, heart rate, weight, height, BMI or head circumference. This confirms to part of the literature (6,7). Next to studies where no associations between LCPUFA supplementation and cardiovascular and anthropometric were found, some studies did report associations, both positive and negative (8-11). This inconsistency is most likely due to the large heterogeneity in study methods (see Table 1 chapter 4).

Important in interpreting the results of the current study is the selective attrition of LF children with a lower cognitive performance on the age of 18 months. This indicates that the results of the LF group could in reality be more negative. Another important factor to take into account is the relatively short supplementation period of 2 months. The Groningen LCPUFA study was part of a set of three supplementation studies. Each study featured a different supplementation period, either 2, 4 or 6 months. One may argue that a 2 month period is too short, taking into consideration that the infant in this period is to be exclusively formula fed and has a continued need of DHA and AA until at least the age of 16 weeks. At that point the ability of the infant to synthesize its own DHA covers about its own need (12). Taking this short period into consideration it is important to realize that the results coming from this study may present an underestimation of the supplementation effect, if supplied during the entire period until weaning. In this respect the review of Simmer (13) is important. The review assessed a large set of studies that evaluated the effect of LCPUFA supplementation. In this large set the supplementation period ranged from 2 months (the current study) until over 1 year. The results from the studies that supplemented for a short period did not differ from those supplementing a longer period. Simmer concluded that no beneficial effects of LCUPFA supplementation were found (13). However, looking carefully and taking into account age at outcome, a slightly different picture arises. Studies evaluating the effect of supplementation before the age of 4 months did find an advantage for DHA supplementation, studies evaluating this effect after the age of 4 months did not (14).

6.2 Effects of breastfeeding

In literature, general positive effects of breastfeeding on neuro-motor development (15) and cognitive development (16-19) have been reported. An important role in this advantage of breastfeeding over formula feeding is played by social economical factors such as maternal IQ and social economic status, the advantages of breastfeeding become attenuated when adjusted for these variables (20-22). Which is the reason that in the current thesis all findings were adjusted for a series of factors among which a proxy of maternal verbal IQ and ses. A second possible explanation for the advantage of breastfeeding over formula feeding is the nutritional difference. One of these differences is the presence of LCPUFA. This is present in human milk and until recently not in formula (23).

Building on the above mentioned assumption that the presence of LCPUFA plays a role in the advantage of breastfeeding over formula feeding, it is expected that regions benefitting from LCPUFA supplementation (1) would be the ones demonstrating a breastfeeding advantage. These are in particular circuitries involved in sensorimotor integration, motor learning, intelligence, executive functions, attention, and memory. Breastfed children were found to demonstrate a slightly better development than formula fed chil-

dren, they demonstrated less fine manipulative dysfunction when compared to both formula groups and also improved IQ was found in those children that had been exposed to smoking during pregnancy when compared to the un-supplemented formula group. These differences point towards the circuitries mentioned above, however one could have expected more differences. This dissociation between our findings and our expectations may be explained by the same factors that have discussed before: nutrition is but one of the many factors influencing neuronal development, not all regions in the neuronal network profit equally from the higher LCPUFA levels and there is fundamental difference between the developing baboon brain (on which a large part of our expectations are based) and the developing human brain.

Smoking during pregnancy is found to be an interacting factor, similar to the supplementation effect reported in an earlier section. The IQ of children exposed to smoking during pregnancy was higher when breastfed in comparison to children fed control formula. This suggests that breastfeeding, containing LCPUFA, compensates for the diminished LCPUFA levels of children that is a result of maternal smoking during pregnancy (4). This corresponds to the work of Batstra (5), who showed that the negative effects of maternal smoking during pregnancy on cognitive development at age 9 were countered by breastfeeding.

Generally speaking, in the current thesis a less than expected number of neuro-motor and cognitive abilities were found to differ between breastfed and formula fed children. Based on the literature differences were expected in the field of general neurological development (15) and general cognitive development (16,19), in the latter case most notably IQ (17,18). An important role in this respect may be played by the adjustment in all analyses for a series of factors among which a proxy of maternal verbal IQ and social economical status, as mentioned earlier this is known to attenuate differences between formula fed and breastfed infant development.

In addition to neuro-motor and cognitive development, attention was also paid to cardiovascular and anthropometric development. No associations were found with blood pressure. In an important review, presenting the blood pressure differences between groups of breastfed versus formula fed infants, Martin (24) concluded that these groups indeed seemed to differ. A pooled difference between breastfed and formula fed infants of 1.4 mm Hg systolic blood pressure and 0.4 mm Hg diastolic blood pressure was found after the age of 12 months. A difference of this magnitude could also be expected in the current study. Unfortunately our study was underpowered to detect such a subtle difference. Post-hoc power analyses using a $\alpha = 0.05$ and a power of 0.8 indicated that the study was capable of detecting a difference of minimally 1.5 mm Hg. This means that with regard to blood pressure we can only conclude that there is no difference between the groups of more than 1.5 mm Hg. As a second cardiovascular parameter we measured heart rate. A lowering of 2 beats per minute was found in the breastfed group. Resting heart rate may be considered to be a simple measure of vagal function (25). A minor decrease in heart rate may

therefore indicate a shift in autonomic control in which there is a minor increase in vagal dominance. The latter is known to be associated with decreased risk of diabetes, hypertension and mortality in later life (25).

The anthropometric development of the children was measured in body length, weight (resulting also in BMI) and head circumference. No differences between breastfed and formula fed children were found. Based on the literature a small reduction of obesity in later childhood could be expected (26,27). Also with respect to the anthropometric parameters our study was slightly underpowered; using $\alpha = 0.05$ with a power of 0.8 the study was capable of detecting a difference in weight of 1.5 kg and a difference in height of 1 cm. This means that subtle differences could not be detected. Obesity development is known to be related to length of breastfeeding. In the current study 53% of mothers gave maximally 8 weeks full breastfeeding. This is a relatively short period, in that it is much shorter than the length of 6 months exclusive breastfeeding as advised by the WHO (28), however it is representative for the Dutch feeding practices (29).

6.3 Effects of neonatal LCPUFA status

The neonatal evaluation in the current study evaluated DHA, AA and transfatty acid levels in the umbilical cord and its associations with development at age 9.

Fatty acid levels in the vessel walls of the umbilical vein and artery reflect nutritional supply and use during pregnancy. The umbilical vein transports the blood from the mother to the foetus and the umbilical artery transports the blood from the foetus back to the mother. Therefore fatty acid content of the vein can be seen as a reflection of nutritional supply and the fatty acid content of the artery as a reflection of foetal use. Higher DHA levels at birth were found to be related with a better neuro-motor condition, this confirms to literature (30,31) and is in line with our previous results at the age of 18 months (32).

Looking more specifically to the separate neuro-motor domains we found that in particular the domains choreiform dyskinesia and posture and muscle tone were found to be related to DHA levels. These domains are mediated predominantly by the subcortical circuits in which the striatum plays a central role. These subcortical circuits have been shown to benefit from DHA supplementation (1).

Detailed analyses revealed that the association between venous DHA and general neuro-motor development was restricted to boys and that for arterial DHA no sex difference was present. Sex differences in neurological development have been reported before. A robust finding is a 10% overall larger brain size and cerebellum size (corrected for total brain size difference) for boys (33-38). Also, the developmental trajectories of gray matter volume and cerebellar volume differ between the sexes (gray matter volume peak size in boys at 14.5 years, in girls at 10.5 years; cerebellar volume peak size in boys at 15.5 years, in girls at 12 years (39,40). Relevant subcortical structures that show gender related differ-

ences are the caudate (generally found to be larger in girls (38,41-43)), the amygdala (generally found to be larger in males (44-46)) and the hippocampus (found to be larger in females (47)). Both the amygdala and the hippocampus differ in sex steroid receptors, the amygdala features higher androgen receptor levels and the hippocampus features higher estrogen receptors (48,49), indicating a possible hormonal role in neuronal gender differences. The size of the basal ganglia, including the caudate, and the gradual decrease of this structure has been related to neurodevelopmental disorders such as ADHD (50) and obsessive-compulsive disorder (51) for which boys are known to be more sensitive than girls.

In the present study we found a gender difference, the relationship between venous DHA and neuro-motor development was restricted to boys. This may be related to the above mentioned sex difference in brain development as the basal ganglia are also known to benefit from higher DHA levels (1). Not only was there a sex difference with respect to neuro-motor development, there was also a difference in relation between vein and artery. The relationship between DHA and neuro-motor development was present in boys only in the vein and not in the artery. This may indicate that boys are more dependent than girls on supply of DHA for their neuro-motor development, suggesting that DHA-supply may be a bottle neck in their development.

No associations were found between AA levels and any of the outcome measures at age 9. This corresponds to literature were only a relation between prenatal AA levels and development is found at a very young age (52-54).

An unexpected finding was the positive association between trans fatty acid levels, both in vein and artery, and selective attention. There has been little research on the effects of trans fatty acids. The studies that have been done reveal negative associations between trans fatty acids and growth in infants and children (55-59) and negative associations between trans fatty acids and *general* neurological development at young age (32,52). Little is known about the neuronal circuits supporting specifically selective attention (2). However as part of more complex processes, such as cognitive control, areas such as the supplementary motor area, the frontal eye fields, anterior cingulate cortex, dorsolateral prefrontal cortex, ventrolateral prefrontal cortex, lateral orbitofrontal cortex, and the temporal and parietal regions, have been mentioned (60-62). The association between selective attention and transfatty acids suggest that transfatty acids may specifically affect these regions. However, the process of cognitive control, and its underlying neuronal circuits, are closely related to executive functioning, an outcome not affected by transfatty acids in the current study. The authors know of no biologically plausible explanation for this contradictory finding. No relations were found between trans fatty acids and any of the other indices.

No associations were found between prenatal DHA and cognitive or behavioral outcomes. This confirms to literature, studies reported no associations between prenatal DHA and general cognitive development at 18 months (32,63), at 2.5 years (64) , at 4 years (65) and at 7 years (66,67). There is one exception, a study by Boucher et al. who reported a positive association between neonatal DHA and memory at a mean of 11 years (68). The

discrepancy between the findings of Boucher et al. and the rest of the literature may be explained by differences in the characteristics of the studied groups. In the studies reporting no associations all children were from western low fish consuming communities having received DHA supplementation. The study by Boucher et al. was performed in an Inuit population, which is known for its generally high fish and therefore DHA consumption.

6.4 Attrition

The follow-up at 9 years was completed in 72% of the original population. Long term follow-ups inevitably suffer from attrition. Loss to follow-up depends on length of follow-up, age of the subject, nature and perceived benefit of the test, degree of inconvenience involved and the ability to trace and contact subjects (69). Based on the above mentioned criteria the current study could be expected to suffer from high attrition: the follow-up period is long, a high percentage of children had moved from their original address, the perceived benefit of the tests are low (the population being healthy) and the session was a lengthy 3 hours. Taking these unfavorable factors into account a much larger attrition might have been expected.

Next to a general attrition, the study also suffered from selective attrition. There was a general loss of children having shown significantly more often normal-optimal general movements at 3 months. Meaning we have a neurologically less optimal group of children remaining in the study. Additionally, restricted to the LF group a larger percentage of boys than girls was lost and also children with a worse cognitive development as measured at 18 months. Taking all factors into account we consider an attrition of 28% favorable, however due to the selective nature of this attrition it is important in the interpretation of the results to be aware of the LCPUFA group being a more positive selection of children than the other two groups. Important in this respect is the comparison of this study to other studies in the field. Interestingly, in the Cochrane review of Simmer et al. the level of attrition of the current study was considered as favorable, allowing for a classification as 'moderate to low risk of bias' (13).

6.5 Conclusions

This thesis shows that LCPUFA supplementation of formula during the first two postnatal months in healthy term infants does not promote neuro-motor condition at school age. Neither seemed LCPUFA supplementation to be associated with a beneficial effect on cardiovascular and anthropometric development. However, indications were found that LCPUFA supplementation affected cognitive development. The effect was dependent on maternal smoking status during pregnancy. LCPUFA supplementation of infants prenatally

exposed to smoking was associated with a beneficial effect on cognition at 9 years, whereas LCPUFA supplementation of infants who had not been exposed to maternal smoking during pregnancy was associated with a minor but statistically significant negative effect on cognition at 9.

Breastfeeding – in comparison to formula feeding – was associated with a slightly better neuro-motor outcome, marginally better cardiovascular outcome and improved cognitive abilities.

Finally the studies included in the present thesis indicate that neonatal fatty acid status had an effect on developmental outcome. Higher DHA levels at birth were associated with a more optimal neuro-motor development at school age and higher trans fatty acid levels were associated with improved selective attention. DHA and AA were not associated with cognition and behavior at 9.

6.6 Future research

The current study is the first to evaluate broadly neurological development at school age after early postnatal LCPUFA supplementation. The supplementation period of two months was relatively short. It would be interesting to investigate whether a longer supplementation period, e.g. 6 months, akin to the advised period in which the infant is exclusively dependent on breastfeeding or formula feeding, would yield different results.

The next step for the current study might be evaluation at puberty. A period of great neuronal and bodily changes which may alter the relationships between early nutrition and developmental outcome (2,33).

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Chapter 7

Summary

This thesis covers two main subjects. Firstly, it investigates the effects of supplementation of infant formula with two Long-Chain Poly Unsaturated Fatty Acids (LCPUFA): the omega-3 fatty acid docosahexaenoic acid (DHA) and the omega-6 fatty acid arachidonic acid (AA) during the first 2 months after birth on developmental outcome at school age. Secondly, the thesis evaluates relationships between fatty acids as measured in the umbilical cord vessel walls and child development at school age.

Women were recruited while pregnant. If a healthy at term infant was born, the infant was enrolled in the study. The majority of participating families donated umbilical cord tissue. The infants who were formula fed were randomly assigned to either of two groups. One group received standard formula (control group) the other group received LCPUFA supplemented formula (LCPUFA group). The study was performed in a double blind fashion, meaning that neither parents nor researchers were informed on group allocation. In addition a group of infants whose parents chose to breast feed, were included in the study as a reference group.

The literature indicates that DHA and AA supplementation of formula in healthy term infants is related to better development during the first 3 or 4 months after birth, but that this effect has disappeared at the age of 1.5–2 years. This does not necessarily imply that the effect has disappeared permanently; it is also possible that with increasing age the effect will be re-expressed. Such an effect is known of breast feeding: absent at 1.5–2 years and present at school age and in adulthood.

No other studies to date have reported on the long-term effects of LCPUFA supplementation of formula on development at 9 years. In the current study, the effects on neuro-motor development, cognitive development, behaviour, body length, weight, head circumference, blood pressure and heart rate have been evaluated.

No differences were found in neuro-motor development between the two formula groups. Effects on cognitive development were found to be dependent on maternal smoking during pregnancy. In the group of children who were born to smoking mothers LCPUFA supplementation was associated with higher scores on verbal IQ and learning and memory. In the group of children of non-smoking mothers LCPUFA supplementation was associated with a lower score on verbal memory. In addition, children who had received LCPUFA supplementation had slightly lower scores on executive functions than the children who had received control formula. The latter effect was not dependent on maternal smoking status during pregnancy. No differences were found between the two formula groups with regards to behaviour, weight, body length, head circumference, blood pressure and heart rate.

Being breastfed was associated with a minor advantage in neuro-motor development and cognitive development. Breast fed children demonstrated slightly better fine manipulative skills than formula fed children. The effect on cognitive development was mediated by maternal smoking during pregnancy. In the children who were exposed to maternal smoking during pregnancy a higher IQ was found in breastfed children compari-

son to formula fed children. The children who had not been exposed to maternal smoking during pregnancy did not demonstrate breastfeeding related cognitive advantages. Also, no differences were found between breast and formula feeding for any of the other cognitive functions, nor for behaviour, weight, body length, head circumference and blood pressure. For heart rate a small advantage was found for breastfed children, their heart rate was lower than that of formula fed children.

The second part of the study addressed relationships between fatty acids in the umbilical vessel walls and development at age 9. The fatty acid profile of the umbilical vessel walls may be regarded as a proxy of prenatal fatty acid status. In the analyses a potentially confounding effect of postnatal group allocation was taken into account. The analyses revealed that higher DHA levels in the umbilical vein were related to less choreiform movements, less posture and muscle tone problems and a lower prevalence of the clinically relevant form of minor neurological dysfunction, i.e. the complex form of minor neurological dysfunction. No associations were found between DHA and cognition or behaviour. Also, AA did not show any relations with development at age 9.

To summarize, the data of the present study indicate that a higher prenatal DHA level is associated with a better neurological outcome at 9 years, without affecting cognition and behaviour. Prenatal AA was not associated with developmental outcome at 9. The study was unable to demonstrate a consistent beneficial effect of postnatal supplementation with DHA and AA on outcome at 9 years. The effect of postnatal DHA and AA largely depended on maternal smoking status during pregnancy. With respect to breastfeeding: our study confirmed that breastfeeding is associated with a minor positive effect on neurological and cardiovascular development.

Chapter 8

Nederlandse samenvatting

Dit proefschrift heeft twee hoofdonderwerpen. Als eerste behandelt het het effect van toevoegen van het omega-3 vetzuur docosahexaeenzuur (DHA) en het omega-6 vetzuur, arachidonzuur (AA) via de flesvoeding gedurende de eerste 2 maanden van het leven op de ontwikkeling van het kind op 9 jaar. Het tweede hoofdonderwerp is de relatie van verschillende vetzuren zoals gemeten in de navelstreng met de ontwikkeling van kinderen op 9 jaar.

Zwangere vrouwen werden gevraagd mee te doen aan het onderzoek. Als de vrouwen, bevielen van een gezond op tijd geboren kind mochten de moeder-kind paren meedoen aan het onderzoek. Een aanzienlijk deel van de moeders doneerde na de geboorte navelstreng weefsel. De kinderen waarvan de ouders kozen voor het voeden met flesvoeding werden verdeeld in twee groepen. De ene groep kreeg standaard flesvoeding (de controle groep) en de andere helft kreeg flesvoeding met daaraan DHA en AA toegevoegd (de LCPUFA groep). De verdeling werd door het lot bepaald – dit wordt een Randomized Controlled Trial genoemd. Het onderzoek werd dubbelblind uitgevoerd, dat wil zeggen dat zowel ouders als onderzoekers niet wisten welk kind welke voeding kreeg. Ontwikkeling werd ook in een derde groep bestudeerd, namelijk van kinderen van wie de ouders kozen voor borstvoeding. Deze groep fungeerde als referentiegroep.

Uit de wetenschappelijke literatuur was bekend dat DHA en AA toevoeging aan flesvoeding een positief effect heeft op de ontwikkeling gedurende de eerste 3 à 4 maanden na de geboorte, maar dat de effecten op de leeftijd van 1,5 à 2 jaar verdwenen zijn. Dit hoeft echter niet te betekenen dat het effect helemaal verdwenen is, het is ook mogelijk dat het effect tijdelijk verdwenen is en met de verdere ontwikkeling van de hersenen weer te voorschijn komt. Zo is een effect van borstvoeding ook niet aantoonbaar op 1,5-2 jaar, maar toonde men wel kleine, positieve effecten op de ontwikkeling op de schoolleeftijd en in de volwassenheid.

Geen onderzoek bestudeerde de effecten van het toevoegen van LCPUFA aan flesvoeding op de lange termijn ontwikkeling, dat wil zeggen de ontwikkeling op de schoolleeftijd. Het huidige onderzoek bestudeerde de effecten op neuro-motore ontwikkeling, cognitieve ontwikkeling, en het gedrag, als ook die op lengte, gewicht, hoofdomtrek, bloeddruk en hartslag.

Het huidige onderzoek toonde aan dat er geen verschil was in neuro-motore ontwikkeling tussen de beide flesgevoede groepen. De effecten op de cognitie bleken samen te hangen met het wel of niet roken van de moeder tijdens de zwangerschap. Kinderen van rokende moeders hadden een voordeel van de aanwezigheid van LCPUFA in de flesvoeding voor wat betreft hun verbale IQ en hun leren en geheugen. Kinderen van niet rokende moeders hadden een klein nadeel van de aanwezigheid van LCPUFA in de flesvoeding voor hun verbale geheugen. Verder was de aanwezigheid van LCPUFA bij alle kinderen (of de moeder nu al of niet gerookt had tijdens de zwangerschap) geassocieerd met een licht negatief effect op de planningsfuncties van de kinderen. Er waren geen verschillen aanwezig tussen de beide flesgevoede groepen in gedrag, gewicht, lengte en hoofdomtrek en ook niet in bloeddruk en hartslag.

Borstvoeding hing samen met een klein voordeel in de neuro-motore en de cognitieve ontwikkeling in vergelijking met de flesvoeding. Borstgevoede kinderen hadden iets betere fijne manipulatieve vaardigheden en bij de kinderen die een moeder hadden die gerookt had tijdens de zwangerschap werd een wat hoger IQ gevonden. Voor de kinderen van een moeder die niet rookte tijdens de zwangerschap was er geen verschil tussen de flesgevoede en borstgevoede kinderen. Ook waren er geen verschillen tussen borst- en flesvoeding voor de andere cognitieve functies, noch voor gedrag, gewicht, lengte, hoofdomtrek en bloeddruk. Voor hartslag was er een klein voordeel voor de borstgevoede kinderen, hun hartslag was wat lager.

Als laatste hebben we gekeken naar de effecten van de vetzuren zoals die gemeten zijn in de navelstreng. De vetzuurstatus in de wand van de navelstrengvaten weerspiegelt de prenatale vetzuurstatus. Deze geven een beeld van de vetten in de voeding die het kindje heeft gehad tijdens de laatste periode van de zwangerschap. De concentratie van verschillende vetzuren werd in verband gebracht met de neuro-motore, cognitieve en gedragstoestand van het kind op 9 jaar. In de analyses hielden we rekening met de postnatale voedingsgroepen. We vonden een verband tussen DHA en neuromotore ontwikkeling: meer DHA in de navelstrengader hing samen met minder choreiforme (onwillekeurige) bewegingen, minder problemen in de regulatie van houding en spierspanning en een in zijn algemeenheid betere neuromotore conditie.

Samenvattend kunnen we zeggen dat hogere DHA-niveaus voor de geboorte meer effect lijkt te hebben op de neuromotore ontwikkeling dan DHA toevoeging aan flesvoeding gedurende 2 maanden na de geboorte. Daarnaast kunnen we zeggen dat DHA en AA toevoeging aan de flesvoeding een niet-eenduidig effect lijkt te hebben op de ontwikkeling op 9 jaar. De aard van het effect hangt af van het al dan niet gerookt hebben van de moeder tijdens de zwangerschap. Ons onderzoek bevestigd andere onderzoeken die aangeven dat borstvoeding geassocieerd is met een klein positief effect op de ontwikkeling van hersenen en het cardiovasculaire systeem.

Chapter 9

Dankwoord

Het schrijven van een proefschrift is een proces dat veel tijd en energie kost. In mijn geval wat meer tijd dan vooraf gepland. Maar zoals ik vooraf hoopte is het de tijd zeker waard geweest. Er zijn veel mensen die in deze periode een grote rol hebben gespeeld en zonder wie dit proefschrift er niet was gekomen.

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Prof. Dr. Vaclav Fidler, statistiek is prachtig, een soort van toverdoos gevuld met de meest magische instrumenten waar bij vlagen prachtige en begrijpelijke maar ook bij vlagen volledig onnavolgbare resultaten uitkomen. U hebt het vermogen deze magische instrumenten om te zetten in logische en begrijpelijke processen en daardoor statistiek tot een bruikbaar geheel te maken. Mijn dank hiervoor.

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Curriculum Vitae
and list of publications

Curriculum Vitae

Corina de Jong werd geboren op 29-06-1980 te Heerenveen. Na enkele verhuizingen ging ze op 19 jarige leeftijd in Groningen Psychologie studeren. Groningen zou de rode draad in haar carrière blijken.

Tijdens haar studie lonkte het buitenland en DaimlerChrysler lonkte terug met een afstudeerstage. Stuttgart werd haar nieuwe haven en onderzoek naar de menselijke factor in de auto bracht haar een eerste leuke en interessante stap op de arbeidsmarkt. DaimlerChrysler USA was de volgende. In 2005, na het behalen van haar master titel, deed ze in Portland (Oregon) praktisch onderzoek naar de optimale inrichting van chauffeurscabines. Een mooie, interessante tijd en het leverde uiteindelijk een stevige basis voor het behalen van haar huidige doctoraat. Corina kreeg hiervoor de kans middels het 'Groninger LCPUFA-onderzoek' bij de afdeling OntwikkelingsNeurologie van het Universitair Medisch Centrum Groningen waarvan u hier de resultaten in handen hebt.

Corina kreeg in 2011 haar huidige baan, een postdoc-aanstelling bij de afdeling huisartsgeneeskunde bij het zelfde UMCG. Haar primaire taak bestaat uit het uitvoeren, opzetten en managen van (internationale) onderzoeken met betrekking tot het behandelen van longproblematiek binnen de huisartspraktijk. Daarnaast zette zij gedurende drie jaar haar onderzoek voor de afdeling OntwikkelingsNeurologie voort en heeft dat nu met dit proefschrift afgerond.

Naast wetenschappelijke activiteiten is Corina fanatiek westernruiter en is ze lid geworden van de medezeggenschapsraad van de basisschool waar haar zoon naar toe gaat. Met het afronden van het onderzoek hoopt ze wat meer tijd te krijgen voor deze activiteiten. De wereld ligt aan haar voeten……

List of publications

De Jong C, Kikkert HK, Fidler V, Boehm G, Decsi T, Hadders-Algra M. Neonatal fatty acid status and neurodevelopmental outcome at 9 years. Br J Nutr Submitted.

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